STUDIES IN AMINO ACID UTILIZATION

V. THE DETERMINATION OF MINIMUM DAILY ESSENTIAL AMINO ACID REQUIREMENTS IN PROTEIN-DEPLETED ADULT MALE ALBINO RATS [1]

C. H. STEFFEE, R. W. WISSLER, E. M. HUMPHREYS, E. P. BENDITT, R. W. WOOLRIDGE AND P. R. CANNON

Department of Pathology, University of Chicago

ONE FIGURE

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In previous communications we have shown that a mixture of 16 crystalline and purified amino acids can serve as an excellent substitute for dietary protein in a ration otherwise adequate for adult rats, whether for maintenance (Wissler et al., '48) or for tissue-protein regeneration (Frazier et al., '47). Moreover, the absence from this ration of any one of 9 amino acids is quickly followed, either in normal or protein-depleted rats, by a loss of weight, appetite, and body nitrogen. The 9 amino acids are tryptophan, histidine, lysine, threonine, phenylalanine, methionine, leucine, valine and isoleucine. In other words, each of these amino acids functions as a dietary essential and is required in definite daily amounts in order to maintain normal processes of protein metabolism. Because of this fact one can determine for each of these amino

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acids the daily minimum quantity necessary for over-all tissue protein regeneration. This can be done by feeding isocaloric rations to a series of protein-depleted rats while at the same time keeping all amino acids but one at a constant but adequate level of intake. For example, if the rations are identical except for varying amounts of a particular essential amino acid, these amounts representing 0, 20, 40, 60, 80, or 100% of the standard, it is possible in a 10-day period to observe the consequences of the varying amino acid deficiencies in terms of weight gain and food consumption, and thereby to approximate the minimum amount of each amino acid required in relation to others and to the ration as a whole.

EXPERIMENTAL

In the experiments here reported the following methods were employed: Rats were selected from larger groups of young adult male albino rats (Sprague-Dawley strain) which had been subjected to protein depletion for a period of from two to three months by use of a ration low in protein but adequate in calories, vitamins and minerals (ration 3E, Wissler et al., '48). On such a ration the rats lost from 24 to 33% of their original weights. They also became hypoproteinemic and moderately anemic, with concentrations ranging between 3.25 and 4.99 gm of protein per 100 ml of serum and between 10.0 and 14.8 gm of hemoglobin per 100 ml of blood. The animals were placed in individual cages, and after a 10-hour fast they were weighed and bled in order to determine serum protein and hemoglobin concentrations as a means for choosing animals with fairly comparable serum protein and hemoglobin values. Two days later the animals were again fasted and their weights recorded, after which they were offered 15 gm portions daily of a ration containing 48 cal. and 1.49 gm of the amino acid mixture, calculated as utilizable forms (Wissler et al., '48), minus varying amounts of the amino acid being tested. Diet consumption was carefully recorded each day in order that the intake of a particular amino acid could be calculated.

After the rations had been fed for 10 days the animals were fasted for 10 hours and were then weighed and sacrificed, following which chemical analyses of the liver and, in some instances, of the carcasses were made. The minimum amounts of each essential amino acid required for effective tissue protein regeneration in association with all the other amino acids in the ration were ascertained as follows: Under similar conditions when the standard amino acid ration (ration A, Frazier et al., '47) was fed to a series of 24 protein-depleted rats, the average weight gain for 10 days was 46 ± 5.03 gm. By establishing as a baseline this gain minus one standard deviation, a rat gaining 41 gm for this period was considered to have performed satisfactorily in terms of tissue protein regeneration. Using this criterion, the preliminary experiment in which the critical amino acid was tested in increments of 20% served to ascertain the approximate minimum. In a second experiment, using smaller increments, e.g., 40, 45, 50, 55, and 60% of the standard, the requirement was determined more precisely.

The amino acid requirements as thus determined were then calculated in terms of milligrams per kilogram of body weight. Although it is true that nitrogen requirements for maintenance are a function of body surface area, the requirements for growth or for tissue-protein regeneration (repletion) in the protein-depleted rat cannot be so expressed. Thus, as previously suggested (Benditt et al., '48), we have considered the active protoplasm as a chemical system and have calculated the amino acid requirements in terms of milligrams of amino acid necessary per kilogram of body weight. This is done to permit comparison of repletion requirements with those for maintenance, and also to allow comparison of the repletion performances of rats of varying initial weights on a similar intake of a given amino acid.

The quantity of amino acid utilized per kilogram was computed as follows: For each animal the mean weight for the 10-day period was calculated (the average of the initial and final weights). Knowing the average daily amino acid con-

sumption for the 10-day period, this quantity was then converted to milligrams per kilogram of body weight. As an illustration of the method, the results obtained by feeding varying amounts of tryptophan to 15 protein-depleted rats are presented in table 1. Rat 6 represents the minimum requirement.

From such data, curves were prepared in which the figures for weight gains for the 10-day period were plotted against

TABLE 1

Effect of feeding various levels of tryptophan on weight gain and diet consumption in 15 protein-depleted rats

RAT NO.	INITIAL DEPLETION WT.	WT. GAIN 10 DAYS	AMINO ACID/DAY OFFERED	DIET EATEN	AMINO ACID/DAY EATEN	AMINO ACID EATEN PER KG RAT
	gm	*gm*	*mg*	%	*mg*	*mg*
1	160	49	18	98	17.7	96
2	171	43	18.5	99	18.2	95
3	144	45	16	94	15.1	90
4	165	52	18	91	16.3	85
5	141	39	15	92	13.7	85
6	151	48	15	96	14.4	82
7	146	39	13	96	12.5	75
8	145	35	14	87	12.2	75
9	166	42	15	87	13.1	70
10	145	35	12	95	11.4	70
11	149	22	10	86	8.6	54
12	167	31	12	80	9.6	52
13	165	21	12	76	9.1	52
14	143	3	11	52	5.8	40
15	169	4	9.2	68	6.3	37

those representing milligrams of amino acid consumed per kilogram of body weight. The point at which the smoothed curve intercepted the base line (41 gm of weight gain) was taken as the minimum daily requirement. Figure 1 demonstrates for lysine an essentially straight line relationship between amino acid intakes and weight recovery. It is of interest, however, that the weight curve above the intercept quickly flattens. This suggests that the minimum and optimum requirements for a particular amino acid almost coincide, and

that ingested amounts of a particular amino acid above the minimum presumably are not utilized efficiently for purposes of tissue synthesis under the restrictive conditions of a fixed intake of all the other essential amino acids of the mixture in the basal ration. The findings for all the minima as thus determined are recorded in table 2. For the determination of these values 247 protein-depleted rats were used.

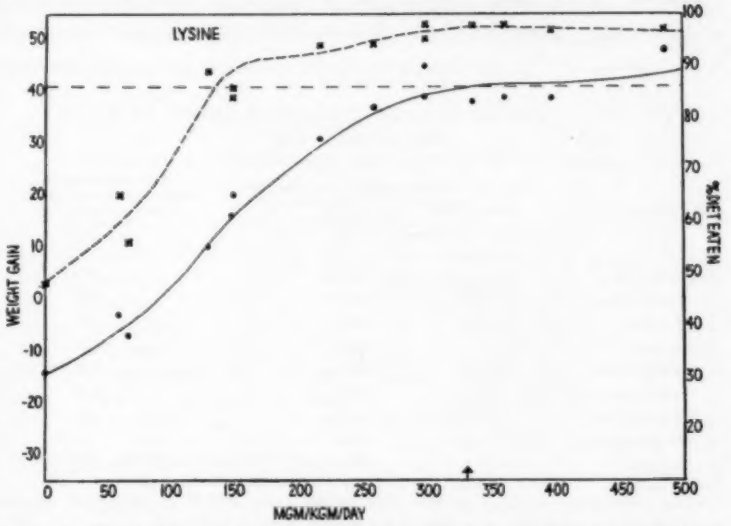

Fig. 1 The influence of various amino acid levels on weight gain and food consumption of protein-depleted rats. Key: Solid line = weight gain curve; dotted line = food consumption curve; · = weight gain, single rat; X = food consumption, single rat; ↑ = minimum requirement for optimum weight gain.

The interrelationship of food consumption with degree of amino acid deficiency was also studied. As a baseline we used the mean percentage consumption of 24 rats receiving ration A (Frazier et al., '47) minus one standard deviation (92.7 ± 6.92). Curves were drawn in which the percentage of ration eaten was plotted against daily amino acid intake and the point of interception with the line representing a ration consumption of 86% was taken as the minimum amino acid re-

quirement. Although in some instances there was a fairly good agreement between food consumption and maximum weight gain with a given amount of a particular esssential amino acid being evaluated, there were also marked differences, particularly with respect to lysine and methionine. These results are shown in table 2 and by the dotted line in figure 1. It is probable, however, that the figures for weight recovery more accurately indicate minimum amino acid "requirements" than do those demonstrating variations in food

TABLE 2

The daily requirements in mg/kg of the protein-depleted rat for each essential amino acid

AMINO ACID	FOR RECOVERY OF WEIGHT	FOR PERCENTAGE CONSUMPTION OF RATION
	Calculated minimum [1]	Calculated minimum [1]
Histidine	120	110
Isoleucine	345	340
Leucine	415	375
Lysine	330	135
Methionine	220	120
Phenylalanine	255	235
Threonine	245	185
Tryptophan	80	60
Valine	290	235

[1] Determined by plotting weight gain or per cent of diet consumption against amino acid consumption; see text and figure 1.

consumption alone. For example, analysis of some of the rat carcasses for water, extractable lipids, protein and ash, was carried out on 14 rats receiving varying quantities of threonine or leucine. Using Fisher's methods, the coefficient of correlation between weight gain and gain in carcass protein was found to be 0.96, and the probability that this result was due to chance selection of the animals was far less than 1 in 1,000 (t value over 12).

Finally, a mixture of amino acids was prepared containing the minimum amounts of each of the 9 essential amino acids as established above in terms of weight recovery. This mix-

ture, together with the customary amounts of non-essential amino acids, was incorporated into the basal ration and the new ration called MEAR (minimal essential amino acid ration, table 3). The amounts of each essential amino acid added to the mixture were calculated to be adequate for a protein-depleted rat weighing initially 144 gm, having a mean repletion weight of 165 gm, and consuming 95% of the ration. Rats fed this ration gained approximately 40 gm in 10 days, and analyses for liver fat showed normal percentages. Also diet consumption was practically complete.

TABLE 3

Milligrams of amino acids per 15 gm of diet per rat per day

AMINO ACID	RATION MEAR [1]	AMINO ACID	RATION MEAR [1]
L(+) Histidine HCl	29.0	DL Valine	102.0
DL Isoleucine	122.0	DL Alanine	86.6
L(+) Leucine	72.5	L(+) Arginine HCl	76.7
L(+) Lysine HCl	79.5	DL Aspartic acid	97.4
DL Methionine	39.0	L(—) Cystine	5.5
DL Phenylalanine	45.0	L(+) Glutamic acid	366.5
DL Threonine	86.0	Glycine	7.8
DL Tryptophan	14.5	L(—) Tyrosine	98.9

[1] Minimum essential amino acid ration.

In a few instances a difficulty of interpretation arose when the weight gain was slightly less than 41 gm in 10 days. Here data from liver analyses helped to validate the other findings. For example, the basal ration used throughout protein depletion as well as for the 10-day experimental period contained 4% of fat and 0.2% of choline chloride. During the process of protein depletion the percentage of liver fat rises until approximately 30% of the original weight of the rat has been lost, following which the percentage gradually falls to 8% or less, which we have found to be normal for a diet with these quantities of fat and choline (Jaffé et al., '49).

Depleted rats, however, are usually used for repletion experiments when the liver fat is above this normal percentage;

but after repletion with ration A the content of liver fat is within normal limits by the 10th day. We do not know as yet how much earlier this may be true. When, on the other hand, a repletion diet deficient in one or more essential amino acids (excepting methionine, tryptophan or valine) is fed, the liver fat, in our experience, is above normal values on the 10th day of repletion. This effect has been most marked with deficiencies of lysine or threonine. Therefore, when percentages of liver fat were above normal values, we assumed the amino acid intake was too low.

It is obvious that rats undergoing repletion have both a basal nitrogen requirement for each essential amino acid and a requirement for refabrication of lost tissue proteins and that, in consequence, the quantity of amino acids in a mixture necessary to produce optimum or maximum repletion is a composite of two factors, viz., (1) the quantity necessary to construct new tissue and (2), the quantity necessary for maintenance of the continuing metabolism. From the results of the present and our preceding observations (Benditt et al., '49) it is evident that the requirements for maintenance and for repletion are of different magnitudes. Furthermore, the relative quantities of each essential amino acid are different. In order, therefore, to obtain the essential amino acid requirements for net tissue synthesis it is necessary to subtract the maintenance needs from the gross values determined in the present experiments. For this purpose the values obtained on the well-nourished animals weighing approximately 300 gm were extrapolated to the 150-gm rat, assuming that the maintenance utilization rate is a function of surface area (Brody, '45). The requirement for the 150-gm rat was then converted from a surface area to a weight basis (Lee, '29). Finally, the gross repletion requirements were corrected for the maintenance requirements to yield the net amino acid utilization for tissue protein synthesis. These data are presented in table 4 and represent the actual milligrams of essential amino acid per kilogram of body weight utilized to accomplish repletion, above the needs for maintenance. It is of interest that

for each essential amino acid the avidity of the depleted tissues augmented the amino acid utilization rate from approximately one-fold in the case of isoleucine to more than 4-fold with respect to lysine.

A noteworthy feature of these figures is the proportionality ratio of the different essential amino acids calculated in relation to tryptophan as unity. Thus, with respect to maintenance it can be seen that the highest utilization amounts are for isoleucine, valine, and leucine, with a surprisingly low ratio for lysine. In contrast, in the animals undergoing repletion

TABLE 4

Daily minimum essential amino acid requirements for protein repletion of the protein-depleted rat

AMINO ACID	FOR MAINTENANCE (CALCULATED FOR 150-GM RAT)		FOR REPLETION	
	mg/kg/day	*ratio*	*mg/kg/day*	*ratio*
Tryptophan	30	1.0	50	1.0
Histidine	35	1.2	85	1.7
Methionine	90	3.0	130	2.6
Threonine	85	2.8	160	3.2
Isoleucine	180	5.7	165	3.3
Valine	120	4.0	170	3.4
Phenylalanine	50	1.7	205	4.1
Lysine	60	2.0	270	5.4
Leucine	110	3.7	305	6.1

the highest utilization amounts are for leucine, lysine, phenylalanine and valine. It should be re-emphasized, moreover, that whether for maintenance or for repletion, all rats consumed rations identical in caloric content and in amounts of vitamins, minerals, and amino acids. Therefore the higher degree of utilization of amino acids in the course of repletion demonstrates the greater capacity of the depleted animal to utilize an assortment of amino acids to synthesize tissue protein. In maintenance, on the other hand, due to the lower degree of utilization of a specific essential amino acid — tryptophan, for example — all the other amino acids ingested in amounts above those necessary to interact with this particular amino acid

were presumably deaminated and discarded, or used for purposes of energy. This would suggest again that for maintenance the minimum amino acid intake is also close to the optimum.

This quantitative order of relationship of essential amino acids in high quality proteins is of both theoretical and practical interest. For example, when proportionality relationships for the 9 essential amino acids are calculated from the composition figures obtained from analyses of beef (Greenwood and Kraybill, '47), whole egg, and casein (Block and

TABLE 5

Proportionality ratios of essential amino acids calculated from amino acid composition figures (see text)

	CASEIN HYDROLYSATE	BEEF MUSCLE	DEFATTED WHOLE EGG	CASEIN	RATION MEAR [1]
Tryptophan	1.0	1.0	1.0	1.0	1
Histidine	2.5	3.2	1.4	1.4	1.5
Methionine	2.5	3.9	3.0	1.9	2.8
Threonine	4	4.2	3.3	2.2	3.1
Phenylalanine	4	3.9	4.0	2.9	3.2
Valine	5	6.0	5.0	3.9	3.6
Isoleucine	5	5.3	5.0	3.6	4.3
Lysine	6	8.5	5.0	3.8	4.1
Leucine	8	8.6	6.0	6.7	5.2

[1] Minimum essential amino acid ration — see table 3.

Bolling, '45), one finds that, with tryptophan expressed as unity, the other values are those shown in table 5. Thus, for these high quality proteins, for each part of tryptophan present there are 6 to 8 parts of leucine, 4 to 8 parts of lysine, and so forth, available to be utilized. This proportional relationship may be of considerable practical importance. For example, if muscle regeneration is attempted by dietary means in a depleted individual with a wasted muscle mass, it would seem essential that, in supplying a mixture of amino acids to the atrophic muscles, there should be a similar distribution of essential amino acids. Otherwise, with a diet deficient in lysine,

there would be no chance for the muscles to obtain enough of this essential amino acid to be utilized with the others in the regeneration of muscle protein.

DISCUSSION

These experiments demonstrate that it is possible to establish levels of amino acid turnover in protein-depleted rats below which the animal cannot accomplish effective tissue protein repletion. This limiting effect of a particular essential amino acid deficiency re-emphasizes the urgent demand of tissues for a continuous supply of these non-synthesizeable amino acids if metabolism is to be maintained at its normal rate and if recovery from tissue protein loss is to be attained. In other words, these amino acids are not only essential for adequate growth; they are essential for the continuing metabolism of adult animals as well, whether for maintenance or for tissue protein regeneration It should be emphasized, however, that the values reported here apply only to the conditions of these particular experiments. In other words, the figures for individual essential amino acid "requirements" presumably have but little absolute value; more important are their proportional relationships.

The principle of proportionality patterns of essential amino acids may help to explain, also, the results of other experiments in which this principle has not been followed (see table 6). Thus Albanese and Irby ('43) failed to obtain growth with young rats fed a ration presumably adequate and containing a mixture of amino acids. Analysis of their amino acid mixture, however, indicates that it was relatively deficient in histidine, leucine, lysine, methionine and threonine, in relation to the tryptophan in the ration. The low proportional relationship of leucine, in particular, could explain the limiting effect of the ration regardless of other factors. Moreover, the original figures reported by Rose ('39) when converted to proportionality patterns, may help to explain the fact that in his earlier experiments he failed to get optimum growth with amino acid mixtures.

Albanese and Frankston ('45) obtained growth with an amino acid mixture, but stated that rats eating a protein digest [2] gained more weight than their other experimental animals despite the fact that their amino acid intake was 20% less. This particular amino acid mixture contained an excess of phenylalanine and was relatively deficient in threonine, valine, and leucine. Comparison with the figures for casein, the principal protein source of the digest used, thus explains the differences in weight gain observed; rats eating the disproportionate amino acid mixture did not gain as well as those

TABLE 6

Comparison of the proportionality ratios of amino acids used by various investigators for growing rats

AMINO ACID	ROSE ('39)	ALBANESE AND IRBY ('43)	KINSEY AND GRANT ('44)	ALBANESE AND FRANKSTON ('45)	KORNBERG ('46)	MEAR [1]
Tryptophan	1	1	1	1	1	1
Histidine	2	1.1	2.5	1.5	1.1	1.5
Methionine	3	1.6	3	3.0	1.5	2.8
Threonine	3	1.3	6	1.8	1.7	3.1
Phenylalanine	2.5	3.5	3.5	7.5	3.4	3.2
Valine	3.5	3.6	7	2.5	3.5	3.6
Lysine	5	3.1	6.3	3.8	3.1	4.1
Isoleucine	2.5	3.1	5	2.3	1.8	4.3
Leucine	4.5	2.7	4.5	3.3	4.0	5.2

[1] Minimum essential amino acid ration — see table 3.

receiving better-balanced hydrolysed casein, because their tissue protein fabrication was presumably limited by the three relatively deficient amino acids. Kinsey and Grant ('44) found that a diet with 11.6% of utilizable amino acids gave the same growth rate as a 9% casein diet. From the proportionality ratios, the reasons for this again become apparent. Their amino acid mixture contained relatively excess quantities of threonine, phenylalanine, valine, lysine, and isoleucine, utilization of which was limited by the availability of the other

[2] "Amigen."

essential amino acids; consequently these excess quantities could not be used in the manufacture of tissue proteins. Kornberg ('46), using a low protein diet to deplete weanling rats until they became agranulocytic, reported that 13 out of 16 rats recovered on an amino acid diet. This ration was relatively deficient in methionine, threonine, lysine, isoleucine, and leucine. When he doubled the quantity of amino acids, 12 out of 17 rats died. This raises the question of the effect of amino acid excess. In a preliminary survey, in this laboratory, feeding ration A to which an excess of 4 times the usual quantity of methionine was added resulted in a suboptimum weight gain. Other amino acids in proportional quantities had no deleterious effects on weight gain. The effects of excess quantities of groups of amino acids have not as yet been studied.

These experiments serve further to emphasize the avidity of the tissues of the protein-depleted rat for amino acids The contrast in the degree of utilization of amino acids for maintenance and for tissue repletion also emphasizes the greater needs of the protein-starved animal. In a recent publication (Benditt et al., '48) this fact was stressed particularly in terms of overall protein utilization. The demonstration that the quantitative amino acid "requirements" for optimum gain of weight in the protein-depleted rat are from two to 5 times those for maintenance suggests the advisability of feeding relatively larger quantities of amino acids or of proteins to patients who have undergone considerable losses of tissue protein.

SUMMARY AND CONCLUSIONS

The quantitative "requirements" for each of the 9 essential amino acids for effective repletion of the protein-depleted rat have been determined experimentally in terms of the ability of the animal to regain lost weight in a period of 10 days. Carcass and liver analyses helped to validate the findings. Although the results obtained have no absolute value except under these experimental conditions, the proportionality re-

lationship of the amino acid utilization amounts appears to have a broad significance. Our conclusions are:

1. The rat that is being repleted utilizes daily from two to 5 times the quantities of essential amino acids utilized for maintenance. Of all these the quantities of leucine, lysine, isoleucine and valine are greatest. These amino acids are also found in high concentrations in mammalian muscle.

2. During repletion, changes in the gross weights of the animals directly parallel gains in carcass protein, indicating that the former give a good indication of amino acid utilization in the processes of tissue-protein synthesis.

3. A dietary deficinecy of certain essential amino acids, notably lysine, threonine and leucine, results in a fatty liver even after 10 days of repletion. This indicates, presumably, an inability of the protein-depleted rat to metabolize the abnormal amounts of liver fat present at the beginning of the experiment.

4. These experiments suggest that the variant results obtained by other investigators, using amino acid rations, may be explained by their use of inadequately proportioned mixtures of amino acids.

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HUMAN MILK STUDIES

XXVII. ESSENTIAL AMINO ACIDS IN HUMAN COLOSTRUM AND TRANSITIONAL MILK[1,2,3]

SOL MILLER, VERA RUTTINGER, MARJORIE MACY RUTLEDGE, ROBERT FRAHM, SHIRLEY MAURER, ELSIE Z. MOYER, MILDRED KAUCHER AND ICIE G. MACY

Research Laboratory of the Children's Fund of Michigan, Detroit

J. P. PRATT AND B. M. HAMIL

Departments of Obstetrics, Gynecology and Pediatrics, Henry Ford Hospital, Detroit, Michigan

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The mammary secretion of a healthy mother is generally acknowledged to possess nutritive qualities which provide advantages for her infant, although the factors responsible for its superior value have not been defined clearly. In a recent publication, Macy ('49) summarized all data obtained by this laboratory relating to the constituents of human colostrum and milk obtained from healthy women under highly controlled conditions of dietary, collection and analysis; and, on behalf of the Committee on Maternal and Child Feeding

[1] A preliminary report of a portion of this material was presented before the Fifty-first Annual Meeting of the Michigan Academy of Science, Arts and Letters, Ann Arbor, Michigan, March 21–22, 1947.

[2] The investigation represented in part by this paper was partially supported by a grant from the Nutrition Foundation, Inc., and was made possible by the cooperation of J. P. Pratt, M.D., Chief of the Department of Obstetrics and Gynecology, who selected the subjects and provided prenatal and obstetric care; Brenton M. Hamil, M.D., Department of Pediatrics, who provided medical care for the infants; Elizabeth Moran, R.N., Director of Nurses, who arranged special nursing service for the subjects; and Annie Lou Wertz, B.S., Dietitian, who aided in planning the dietary and securing the food samples for analyses, all of the Henry Ford Hospital, Detroit.

[3] The authors are indebted to Max S. Dunn, Ph.D., Chemical Laboratory, University of California, Los Angeles, for the brochure of his microbiological methods prior to publication and for a sample of proline; and to the Rockefeller Foundation and Melville Sahyun, Ph.D., who provided other samples of amino acids.

of the Food and Nutrition Board, National Research Council, this author ('50) has prepared a comprehensive compilation of the values reported in the literature for 20 properties and 254 constituents of human, goat, and cow milks. The protein fraction of Nature's food for infants is of special interest in that its components, the amino acids, are essential for the formation of protein in an infant's body.

Quantitative determinations have established the total protein content of whole milk but relatively little has been done in defining the character of the protein. Previously, Plimmer and Lowndes ('37), Beach, Bernstein and Macy ('41), Beach, Bernstein, Hoffman, Teague and Macy ('41), and Williamson ('44) have employed chemical methods of analysis based on isolation of protein from whole milk followed by separation of each amino acid from the hydrolyzed protein. Microbiological methods have advantages in amino acid analyses, since they permit determination with small amounts of material and eliminate the need for purification procedures (Hodson and Krueger, '47). Block and Bolling ('46) used both chemical and microbiological methods for determining amino acids in the protein of pooled samples of human colostrum and mature milk.

In the present work microbiological methods have been used to determine leucine, isoleucine, valine, histidine, lysine, methionine, phenylalanine, arginine, threonine, and tryptophan in the milk of 10 healthy women (Macy, Williams, Pratt and Hamil, '45) during the first 10 days post partum. Although collections of colostrum were obtained from some of the women for additional days during the puerperium, the analytic material from some samples was not sufficient for all analyses. Subsequent papers will present values for the same amino acids in mature human milk.

EXPERIMENTAL

Samples

The samples analyzed represented composite collections for 38 24-hour periods, each obtained by completely emptying

both breasts at 4-hour intervals. The secretion was manually expressed (Davies, '45), by two nurses trained in the technique, under carefully controlled conditions (Kaucher, Moyer, Richards, Williams, Wertz and Macy, '45; Macy, Williams, Pratt and Hamil, '45). Throughout pregnancy and delivery the mothers had been under the same obstetric supervision and during the puerperium they received diets similar in

TABLE 1

Physical characteristics and daily energy and protein intakes of women who provided colostrum samples

SUBJECT	AGE	HEIGHT	WEIGHT	AVERAGE WEIGHT FOR HEIGHT [1]	OVER- OR UNDER-WEIGHT	GRAVIDAE	ENERGY INTAKE PER DAY	PROTEIN INTAKE PER DAY
	years	*cm*	*kg*		*%*		*Cal.*	*gm*
L. F.	31	170	92	60.1	53	5	2874	106
V. G.	29	155	61	51.4	19	2	2460	97
V. L.	32	160	48	53.1	— 10	2	3266	118
C. O.	27	168	61	58.2	5	2	2354	95
J. M.	26	157	54	52.0	4	2	3340	115
V. S.	35	174	74	62.3	19	3	3376	118
G. S.	28	165	64	55.9	14	2	2947	85
M. S.	25	157	46	52.0	— 12	3	2428	94
E. L.	18	169	64	59.2	8	1	2741	99
F. W.[2]	26	178	68	64.0	6	1	2767	104

[1] McCloy ('38).

[2] Negro.

quality. One of the women (F.W.) was a Negro, the others white. Eight subjects were multiparas who had successfully nursed former children. The general physical characteristics of the 10 subjects are given in table 1, with their calorie and protein intakes. Obviously the actual requirements of women while relatively inactive in the hospital during the puerperium are far lower than at any other time during the milk-producing period.

Analytic material

The hydrolysis method of Stokes, Gunness, Dwyer and Caswell ('45) was used in the digestion of samples for assays of leucine, isoleucine, valine, histidine, lysine, methionine, phenylalanine, arginine, and threonine.

One gram of the dried (Desivac) sample material was digested with 10 ml of 10% hydrochloric acid (prepared from distilled, constant-boiling hydrochloric acid) in sealed pyrex test-tubes in an autoclave at 15 pounds pressure and 120°C. for 10 hours. After being cooled to room temperature the hydrolysates were filtered through Whatman no. 1 paper and the precipitate washed thoroughly with distilled water. The filtrate was adjusted to pH 4.6 with 6 *N* sodium hydroxide, diluted to 200 ml, refiltered through Whatman no. 42 paper, placed in glass-stoppered Erlenmeyer flasks covered with a layer of benzene, and stored in a refrigerator.

An alkaline hydrolysis method developed in this laboratory (to be published) was used for preparing the samples for tryptophan analyses. Hydrolysates to be assayed were diluted, on the basis of the total nitrogen of the original sample, to contain 25 to 34 mg nitrogen per liter for leucine, isoleucine, and valine; 37 to 53 mg nitrogen per liter for histidine and phenylalanine; 51 to 64 mg nitrogen per liter for lysine and methionine; 49 to 72 mg nitrogen per liter for arginine and threonine; and 131 to 183 mg nitrogen per liter for tryptophan.

Assay standards

Both the DL- and L- configurations of amino acids were used in the preparation of standards. The DL- forms, when assayed simultaneously with the L- forms, gave values corresponding to one-half the activity of the latter. Commercially prepared amino acids to be used for standards were first tested for purity and microbiological activity. From preparations checked for potency and purity, standard stock solutions were prepared to contain, per milliliter, 1 mg of each of the 10 amino acids which had been dried in a vacuum oven at

50°C. The solutions were stable for at least a year stored in a refrigerator in amber, glass-stoppered bottles under a layer of benzene. The concentration ranges of the standards, organisms, and media used for each amino acid determination are given in tables 2 and 3.

TABLE 2

Composition of media used in amino acid assays [1]

CONCENTRATIONS PER LITER IN FINAL ASSAY SOLUTION

Component	Media			Component	Media		
	A	B	C		A	B	C
DL alanine (gm)	1	1.5	0.2	L tryptophan (mg)	20	30	50
L arginine (mg)	350	525	100	L tyrosine (mg)	40	60	100
DL aspartic acid (mg)	150	225	200	DL valine (mg)	40	60	100
L cystine (mg)	62.5	94	200	Glucose (gm)	20	20	20
DL glutamic acid (mg)	300	450	800	Sodium acetate (gm)			
Glycine (mg)	40	60	100	(anhydrous)	8	6	3
L histidine (mg)	20	30	100	Ammonium chloride (gm)	8	6	6
DL isoleucine (mg)	40	60	100	Folic acid [2] (μg)	4	4	5
DL leucine (mg)	40	60	100	Solution [3] Adenine (mg)	12	12	5
L lysine·HCl·H_2O (mg)	80	120	100	Solution [3] Guanine (mg)	12	12	5
DL methionine (mg)	30	45	100	Solution [3] Uracil (mg)	12	12	5
DL norleucine (mg)	100	150		Vitamin solution [4] (ml)	10	10	8
DL norvaline (mg)	100	150		Salts A [5] (ml)	5	5	5
DL phenylalanine (mg)	40	60	100	Salts B [5] (ml)	5	5	5
L proline (mg)	40	60	100	Pyridoxamine, 2HCl (μg)			400
DL serine (mg)	150	225	100	Succinic acid (gm)			10
DL threonine (mg)	200	300	100	Asparagine (mg, natural)			266
				Glutamine (mg, natural)			80

[1] The amino acid to be assayed is omitted from the media.

[2] "Folvite," supplied through courtesy of Dr. Benjamin W. Carey, Lederle Laboratories, Pearl River, N. Y.

[3] Adenine, guanine, uracil combined in one solution (Lewis, '42).

[4] Solution containing 50 mg thiamine hydrochloride, 80 mg pyridoxine hydrochloride, 100 mg DL-calcium pantothenate, 100 mg riboflavin, 100 mg nicotinic acid, 5 mg *p*-aminobenzoic acid, and 0.25 mg biotin. All components but riboflavin and biotin were dissolved in water. Riboflavin was dissolved in 0.02 *N* acetic acid and heated. The biotin was crystalline biotin (Merck) dissolved in 50% ethanol. The combined solution was diluted to 500 ml with H_2O and ethanol to give a final concentration of 50% C_2H_5OH.

[5] Snell and Wright ('41).

Technique

The method used in the present work is based upon the procedure developed by Dunn and co-workers and described fully in a brochure (Dunn, '46). The standards and samples were pipetted in triplicate in quantities of 0.2, 0.4, 0.6, 0.8, and 1.0 ml, 2 ml of media were added, and the final volume

TABLE 3

Organism, media [1] and range of standards for amino acid assays

ORGANISM AND MEDIA	RANGE OF STANDARDS
	μg/ml
L. arabinosus 8014 [2]	
Media A	
Leucine	0–50
Isoleucine	0–50
Valine	0–50
L. mesenteroides 8042 [2]	
Media B	
Histidine [3]	0–15
Lysine [3]	0–75
Methionine	0–15
Phenylalanine	0–25
S. faecalis 8043 [2]	
Arginine [3]	0–40
Threonine	0–50
Tryptophan	0–7

[1] Compositions of media are given in table 2.

[2] American Type Culture Collection, Georgetown University Medical School, Washington, D. C.

[3] L- form used; DL- forms of all other amino acids were employed.

was adjusted to 3 ml with water. A glass bead was placed in each tube to facilitate uniform suspension of organisms after growth. Since three dilutions of standards were used, 45 assays were made for each standard and 15 for each sample. The assay was autoclaved 5 minutes, cooled to room temperature, inoculated, and then incubated 16 to 20 hours at 34°C., after which the racks were shaken mechanically

for approximately three minutes, then allowed to rest momentarily to disperse any bubbles. The quantitative response of the organism was measured turbidimetrically with a photelometer and values for the samples were obtained by interpolation from curves plotted for the amino acid standards.

Controls were maintained throughout all assays to assure the validity of each individual determination. A test mixture of pure amino acids, vitamins, and salts was used with every series as a "known," the percentage recovery of the amino acid in the test mixture being calculated for each assay. In addition, a sample of casein and a composite sample of mature human milk were hydrolyzed with each group of samples and assayed simultaneously. An average value was established for each amino acid of casein and human milk. Any assay in which the value for the amino acid content of the controls and duplicate samples failed to check within $\pm 5\%$ was considered doubtful and not included in the results. Also, any assay which failed to maintain an acceptable rate of growth for 20 hours was repeated.

RESULTS AND DISCUSSION

Valid conversion factors for the protein of human colostrum and milk have not been established, although amino acid data have been reported in terms of 16 gm of protein nitrogen per 100 gm protein (Block and Bolling, '46) and in terms of percentage of total protein (Kuiken and Pearson, '49). The assumption that proteins contain 16% nitrogen has been criticized by Vickery ('45), and data expressed as percentage of total protein involve the use of doubtful conversion factors (Jones, '41). For these reasons table 4 presents, in milligrams per 100 ml of secretion, the concentrations of the 10 essential amino acids in each of 38 samples of colostrum and transitional milk obtained from 10 women, 2 to 10 days post partum, with the corresponding volumes of secretion during 24 hours and the concentrations of total solids and total nitrogen. The values obtained by Block and Bolling ('46)

TABLE 4

Essential amino acid in human colostrum and transitional milk
(Milligrams per 100 ml of secretion)

	POST PARTUM DAY	24-HOUR VOLUMES	TOTAL SOLIDS	TOTAL NITROGEN	LEUCINE	ISO-LEUCINE	VALINE	HISTIDINE	LYSINE	METH-IONINE	PHENYL-ALANINE	ARGININE	THREO-NINE	TRYPTO-PHAN
		ml	*gm*											
L.F.	4	1122	13.0	306	145	94	100	35	100	26	62	64	76	30
	5	1441	12.9	313	148	92	98	35	95	23	60	62	75	27
	6	1501	13.0	312	155	96	100	38	106	22	61	63	77	27
	7	1596	13.0	321	139	103	113	40	119	26	65	69	85	30
	8	1638	13.3	318	128	105	106	38	118	26	64	67	83	27
	9	1676	13.2	318	137	97	107	39	119	24	65	70	88	28
	10	1872	13.5	314	119	94	98	34	95	24	58	64	83	28
V.G.	4	1413	11.5	292	147	88	100	40	104	20	63	64	75	30
	5	1471	12.8	286	155	95	109	42	111	20	65	70	80	31
	6	1782	13.1	281	167	104	110	44	112	25	71	71	85	30
	7	1630	13.5	295	169	105	111	44	123	28	69	67	79	30
	8	1895	13.4	284	156	98	96	41	117	22	63	55	75	27
	9	1828	13.0	266	149	94	94	39	107	21	59	60	80	27
	10	1770	13.2	266	137	86	90	37	99	22	54	56	72	27
V.L.	4	794	13.2	307	164	99	112	42	133	19	71	74	87	25
	5	844	13.5	313	157	104	109	44	139	25	74	72	86	31
	6	955	13.9	331	176	112	112	45	141	26	74	69	86	33
	8	1098	14.4	323	188	101	117	45	148	28	71	67	85	32
	9	1118	14.0	318	165	107	110	43	138	24	63	60	80	28
	10	1200	14.0	293	153	99	97	40	137	25	64	57	80	26
C.O.	3	335	11.6	371	214	115	149	46	118	36	84	96	104	41
	4	595	12.3	371	194	109	145	43	119	32	76	84	86	35
	6	821	13.0	322	133	105	122	40	135	25	71	89	96	
	7	798	12.9	322	174	99	115	41	103	30	66	70	77	31
	8	950	12.9	280	162	94	112	38	97	34	61	62	68	29
	9	931	12.8	280	149	88	104	34	91	23	58	56	65	27
J.M.	2	385	11.7	352	174	94	130	44	112	24	79	90	98	42
	5	1121	13.4	352	191	110	132	41		30	70	71	76	33
	6	1125	13.2	315	164	98	108	38	116	24	66	69	82	30
	7	1287	13.2	300	144	92	105	34	106	21	61	61	80	28
	10	1336	12.5	273	113	95	94	30	106	21	51	57	68	
V.S.	8	837	14.2	325	197	121	136	44	122	26	71	71	91	32
	9	932	14.2	317	168	108	117	44	124	26	66	66	86	29
	10	924	14.3	323	170	112	116	40	123	25	69	73	80	30
G.S.	7	1017	12.1	249	130	77	77	29	93	16	48	48	61	23
M.S.	7	880	12.3	261	124	74	86	34	86	18	53	58	70	27
E.L.	8	688	12.5	248	128	73	97	32	88	17	55	50	65	23
F.W.	7	953	14.2	260	123	70	91	35	101	19	60	69	78	25

represented the amounts in the milk protein but those in table 4 include "free" as well as combined amino acid.

Volumes of secretion per day at different intervals post partum varied widely among the individual women, and concentrations of total solids and nitrogen were not directly related to volume of secretion per day. The ranges for all determinations indicate general conformance to a pattern of composition, with total solids ranging from 11.5 to 14.4 gm, averaging 13.1 gm, and total nitrogen from 248 to 371 mg, averaging 305 mg, per 100 ml of secretion. The biologic individuality of the women is shown by the ranges among data for the same mother on different days post partum. These ranges are narrower than the ranges for the group of women. Individual differences are also shown by the variations between levels of total solids and total nitrogen. Values for 4 of 5 subjects who provided samples for 5 or more days post partum show declines in the concentrations of total solids and total nitrogen during the final 2 to 5 days of the puerperium.

The data for the 10 individual amino acids conform with the values for total nitrogen, portraying variability attributable to individual differences among women and among days post partum. Mean values per 100 ml of secretion were: leucine, 155 mg; isoleucine, 98 mg; valine, 108 mg; histidine, 39 mg; lysine, 144 mg; methionine, 24 mg; phenylalanine, 65 mg; arginine, 67 mg; threonine, 80 mg; and tryptophan, 30 mg, with differences between minima and maxima for the individual amino acids varying from 17 mg for histidine to 101 mg for leucine. In general, the greatest concentrations of each essential amino acid were found from the 5th to the 8th day post partum, possibly reflecting physiologic instability in the secretory mechanism; thus re-emphasizing that during the second 5 days of lactation changes in the composition of the secretion from the human breast occur in the direction of mature milk production, and the secretion should not be considered colostrum (Coryell, Harris, Miller, Williams and Macy, '45). Variations in the amino acid composition

of the secretion from different women and from the same woman during different days post partum are obscured in results from analyses of composite samples, and great care must be employed in the collection of a composite to avoid "weighting" of results by inclusion of too many samples from certain individuals or excessive numbers of collections representing certain days of lactation.

Amino acid concentrations in some instances were as high on the second or third day post partum as at the peak within the puerperium. Although the content of total nitrogen or any amino acid in breast secretion per 24 hours is grossly related to the volume produced, the concentration in milligrams per 100 ml is not so related. At any time during the puerperium one mother may supply in a smaller daily volume of secretion an amino acid mixture equal or superior in value to that provided by another mother. An earlier paper from this laboratory (Beach, Bernstein and Macy, '41) reported, for an infant who weighed considerably less than two others, equally satisfactory weight gains on an intake of breast milk less than that received by the other two babies but containing a greater concentration of protein.

The amino acid nitrogen represented by the total mean quantities of the 10 essential amino acids accounted for 37.6% of the average total nitrogen content of the 38 samples of secretion. The mean percentage of total nitrogen accounted for by leucine was 5.4; by isoleucine, 3.4; by valine, 4.2; by histidine, 3.5; by lysine, 7.1; by methionine, 0.8; by phenylalanine, 1.8; by arginine, 7.0; by threonine, 3.1; and by tryptophan, 1.3. The relative distribution of the nitrogen of the individual amino acids in each 100 ml of secretion varied for different women, indicating possible differences in the combination of proteins in their secretions, but showed no trend ascribable to interval in lactation. More fundamental research is needed before the proper factors become available for converting the nitrogen of human colostrum to protein. Furthermore, fractionation studies of the protein

in colostrum are needed to determine whether changes in nitrogen distribution represent changes in protein distribution.

Essential amino acids in "first" and early colostrum

The interest and cooperation of one of the authors (J.P.P.) made it possible to ask each woman in the obstetric ward of the hospital, for delivery or early post partum care, to contribute one or more samples of her milk. Under these conditions it was possible to obtain samples of colostrum representing earlier stages in lactation than any for which data are available in the literature, and the analytical results provide new knowledge of the composition of the initial secretion from the human breast after childbirth.

All samples of secretion were collected by one of the nurses specially trained in the technique of manual expression (Davies, '45), and the composites were accumulated, stored, and handled by procedures previously described (Macy, Williams, Pratt and Hamil, '45). The analytic technique was identical to that described earlier in this paper.

Three composites were obtained for analysis. Composite I included only the first secretion from the breasts after delivery. From approximately 70 women, 133.6 ml of "first" colostrum were accumulated. Samples ranged from a few drops to 4 ml and were collected not less than 8 and not more than 24 hours post partum, depending upon time of delivery and recuperative period in relation to the available time of the nurse obtaining the samples. Collection of a single sample sometimes required the better part of an hour of the nurse's time. Obviously, such samples of the prime secretion — obtained before any spontaneous or nursing loss by a person trained in "stripping" the human breast — also contain maximum amounts of any tissue "debris" from the milk ducts.

Some women contributed only the initial sample but from others a sample was obtained on each of the following two days. In the studies of human milk of which this paper is

a part, the results of all other analyses of colostrum are based on 24-hour collections of secretion from individual women. Those 24-hour collections were *started* with samples of colostrum comparable to those represented in composite I, but these would represent only a small amount of the total sample. This distinction is exceedingly important for comparative purposes because on the basis of it, composites II and III, collected one and two days, respectively, after composite I, actually compare with 24-hour samples usually designated as representing the "first" and "second" days post partum.

The results of analyses of the three composites for the 10 essential amino acids are given in table 5. The concentrations of total solids, total nitrogen and all the amino acids in the "first" colostrum are much higher than any values for human colostrum found in the literature. However, we have not found records of any other analyses representing only the initial fluid obtained from the human breast after delivery. Inspection shows a relatively consistent reduction of as much as one-half in the data for composite II, and a further reduction of about the same magnitude in composite III. The levels of concentration in composite III compare acceptably with those for the 24-hour secretions of individual women during the second and third days post partum (table 4).

The amino acid nitrogen of the 10 amino acids accounted for 45% of the total nitrogen in each of composites I, II, and III, a value considerably higher than the 38% of total nitrogen accounted for by the 10 amino acids in 38 24-hour samples representing the 2nd to 10th days post partum (table 4). The values for the individual amino acids in the composites vary from those in the 24-hour composites, but only for arginine and threonine are percentage nitrogen values in "first" and early colostrum (table 5) significantly different from comparable values in the 24-hour collections (table 4). Arginine in "first" colostrum accounted for 12.1% of the total nitrogen; in composite II (the next day) for 11.6%; and in composite III (a day later) for 10.7%. Threonine in "first" colostrum accounted for 5.4% of the total nitrogen;

TABLE 5

Essential amino acids in "first" and early colostrum

COMPOSITE [1]	TOTAL SOLIDS	TOTAL NITROGEN	LEUCINE	ISOLEUCINE	VALINE	HISTIDINE	LYSINE	METHIONINE	PHENYLALANINE	ARGININE	THREONINE	TRYTOPHAN
	gm	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
							Concentration per 100 ml					
I	17.7	1372	659.6	291.0	571.0	178.5	508.6	90.1	377.4	516.1	629.5	205.2
II	14.4	720	345.7	180.9	298.4	97.8	267.1	53.2	195.9	260.7	317.0	103.6
III	11.8	346	175.5	104.2	140.2	51.6	148.5	30.1	85.7	115.4	129.3	39.9
							Percentages of total nitrogen					
I ("first" colostrum)			5.1	2.3	5.0	3.5	7.1	0.6	2.3	12.1	5.4	2.0
II (first day of lactation)			5.1	2.7	5.0	3.7	7.1	0.7	2.3	11.6	5.2	2.0
III (second day of lactation)			5.4	3.2	4.8	4.0	8.2	0.8	2.1	10.7	4.4	1.6

[1] Volumes of composites were: I, 133.6 ml; II, 274.5 ml; III, 794.9 ml.

in composite II for 5.2%; and in composite III for 4.4%. For the 38 24-hour samples 2 to 10 days post partum, the per cent nitrogen values are 7.0 and 3.1, respectively. The relatively small variation obtained in this work through use of the method employed under rigid conditions of control indicates that the foregoing differences in arginine nitrogen and threonine nitrogen values were actual, but the significance cannot be explained.

SUMMARY

The 10 essential amino acids were determined microbiologically in 38 samples of human colostrum and transitional milk secreted 2 to 10 days post partum. Samples were obtained from 10 women, each representing the composite mammary secretion from both breasts during 24 hours.

The amino acid concentrations per 100 ml of secretion varied with subject and day post partum and did not parallel changes in volume of secretion or concentration of total solids. Total nitrogen and amino acid concentrations followed the same general pattern, with highest values occurring on the 5th, 6th, 7th, or 8th day post partum. The ratios between the nitrogen of the essential amino acids and total nitrogen were not consistent, indicating the possibility of shifting proportions of the casein, lactalbumin, and globulin fractions of the total protein.

The same amino acids were determined in 133.6 ml of "first" colostrum, consisting of from a few drops to 4 ml of the first fluid from the breasts of each of 70 women, 8 to 24 hours post partum. Composites were also analyzed of the colostrum obtained from many of the women the first day and the second day following collection of the "first" secretion. In the "first" colostrum values for total solids, total nitrogen and the 10 essential amino acids were much higher than any values for human colostrum found in the literature. The percentage of total nitrogen represented by the amino nitrogen of the essential amino acids was higher than the corresponding values for the 24-hour collections

of colostrum 2 to 10 days post partum. The greater percentages of amino nitrogen in the composites were accounted for by significantly higher values for arginine and threonine.

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ADDENDUM

After proof of this paper had been returned to the Journal, a paper by Richard J. Block and Diana Bolling appeared in *Archives of Biochemistry* (*25:* 350–353, Feb., 1950) giving the quantities of amino acids in the nonprotein fraction of breast and cow's milk.

THE RETENTION OF FLUORINE WHEN FED AS BONE AND AS SODIUM FLUORIDE

S. H. JACKSON, F. F. TISDALL, T. G. H. DRAKE AND DORIS WIGHTMAN

Research Laboratories of the Department of Paediatrics, University of Toronto, and Hospital for Sick Children, Toronto

TWO FIGURES

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In a previous paper (Drake et al., '49), in which the availability of the calcium of bone was studied, we have pointed out two instances wherein bone has been or is being used as a large scale source of supplementary calcium in the human dietary. The first example was the addition of cooked bone to canned meat products. During and immediately after the last war a large proportion of the canned meat products prepared in Canada for U.N.R.R.A. contained 15% of cooked ground bone. The addition of 0.5% bone meal to all the flour sold in Newfoundland at the present time was given as a second example. This use of bone not only raises the question of the availability of its calcium but also brings into some prominence the study of the amount and retention of the fluorine in edible bone products.

Ellis and Maynard ('36) fed diets containing the same concentration of fluorine to young rats for 8 weeks and determined the concentration of fluorine in the bones at the end of that time. They found that the same amount was retained in the rats' bones when the fluorine was added to the diet as either sodium fluoride or bone meal. They also found that feeding diets containing 8, 12 and 14 p.p.m. of

fluorine, again as either sodium fluoride or bone meal, produced proportional concentrations of fluorine in the rats' bones. The availability of the fluorine in bone meal was thus found to be the same as that of sodium fluoride. Evans and Phillips ('38) fed various bone meals containing different amounts of fluorine and prepared by different methods. They demonstrated only an approximate proportionality between the concentration of fluorine found in the rat femurs and the fluorine in the diet, indicating a difference in availability of the fluorine in the various preparations. This difference they attributed to varying degrees of fineness among the bone meal samples.

On the other hand, evidence has appeared in the literature to indicate that the retention of fluorine from bone meal is less than that from sodium fluoride. Machle and Largent ('43) administered 6 mg daily of supplementary fluorine to an adult man and determined the urinary and fecal excretion. The retention of fluorine was 3.88 mg daily when administered as sodium fluoride in the drinking water, but was only 1.73 mg when supplied as bone meal incorporated in the food. McClure, Mitchell, Hamilton and Kinser ('45) found that absorption of the fluorine of bone meal (i.e., the ingested fluorine minus the fecal excretion) was 64.5% that of sodium fluoride mixed with the food. Greenwood et al. ('46) fed bone meal, defluorinated phosphate and sodium fluoride to puppies for approximately one year. All were fed at a level to supply 5 mg of fluorine per kilogram of body weight. Those puppies receiving sodium fluoride developed dental fluorosis. This abnormality did not develop when the fluorine supplement was in the form of either bone meal or defluorinated phosphate. Furthermore, the teeth were better in those animals receiving bone meal or defluorinated phosphate than in animals receiving sodium fluoride with added calcium and phosphate equivalent to that present in the other supplements.

EXPERIMENTAL

Analytical method

The analytical method employed was based on that of Willard and Winter ('33). The sample was ashed with the use of magnesium acetate as a fluorine fixative, as suggested by Crutchfield ('42). Silver sulfate was added to prevent distillation of chloride (McClure, '39a). The multiple still used for the steam distillation was similar to that described by Churchill ('45). Matuszak and Brown ('45) studied conditions for the titration of fluorine with thorium nitrate. They discarded the buffer commonly employed. We found that the use of a monochloracetate buffer degraded the color of the indicator and that the end-point lost sensitivity. We used the back titration method of Dahle et al. ('38) and Clifford ('40) but eliminated the buffer and carried out the titration after adjusting the solutions to pH 3.0 with 1/50 N HCl.

The recovery of fluorine added as sodium fluoride to various types of materials analyzed during this study was from 93 to 100% complete, with an average of 95%. It was our custom to ash samples in triplicate and to titrate each distillate in duplicate. If the values for the triplicate samples agreed with $\pm$ 5% of the mean, the mean value was accepted as correct. This was usually the case. In those few instances where the values did not agree, further replicates were analyzed until a satisfactory group of checks was obtained.

The problem of obtaining a representative sample of rat carcasses for fluorine analysis was overcome by the following procedure. The carcasses were skinned, the entire gastrointestinal tract removed, then placed in tins and nearly covered with 3% acetic acid. The tins were heated in flowing steam for one-half hour to exhaust gases and were capped while hot. Heating was continued for two to three hours under 15 pounds steam pressure. The carcasses could be kept indefinitely at this stage until it was convenient to proceed with the analysis. The tins were then opened and the contents

homogenized thoroughly in a Waring Blendor. The heating process so softened the bones that they were readily dispersed and an even mixture was obtained. Small samples suitable for ashing were easily weighed out from this mixture.

Fluorine in bone

Experiment 1. A large bone sample,[1] about 200 to 400 pounds at a time, was cooked under steam pressure until soft. The liquor that had been diluted by steam condensation was concentrated by boiling so that the total weight of liquor and bone was the same as the original weight of the sample. The bones and liquor were mixed, ground finely, and canned in 12-ounce cans. These were then autoclaved at 15 pounds of steam pressure for two hours. Samples of commercial classifications of bones were prepared by this method; these were "hard" bones and "soft" bones from beef, veal and pork and samples of beef ribs and vertebra. Attempts were made to prepare a sample of beef shanks but these were so hard the preparation was found to be impractical. Samples were also obtained from a regular commercial run of bone that was being prepared for incorporation in a meat product, and of the meat product containing an additional 12% of this bone. There was also a supply of bone meal, i.e., defatted dried bone ground to a very fine flour.

Table 1 shows the fluorine content of these bones. A number of cans from each batch were analyzed and the fluorine concentration of individual cans was found to be constant for each batch within the limits of error of the method of analysis. A wide variation of fluorine concentration was observed, ranging from 1.16 mg% in veal soft bones to 21.5 mg% in beef soft bones. These figures agree well with the values reported by Evans, Phillips and Hart ('38). These workers found an average of 32 p.p.m. (3.2 mg%) of fluorine in veal bones and 190 p.p.m. (19.0 mg%) in the bones of mature

[1] A number of samples of cooked ground bone were prepared for us by Canada Packers, Ltd.

cows. Bone meal of course was very much higher in concentration, as this was a dried material. Bone for subsequent feeding experiments was drawn from these same batches and the fluorine figures given in table 1 were used for calculating the fluorine present in the form of bone in the various experimental rations.

Fluorine retention from sodium fluoride

Experiment 2. This experiment was undertaken to determine the retention of fluorine by rats fed graded levels of

TABLE 1

The fluorine content of cooked bone and of bone meal

NO.	SAMPLE	FLUORINE
		mg/100 gm
BO – 1	Regular run of bones	4.97
BO – 4	Beef hard bones	14.97
BO – 5	Beef soft bones	21.5
BO – 6	Veal hard bones	1.24
BO – 7	Veal soft bones	1.16
BO – 8	Pork hard bones	7.39
BO – 9	Pork soft bones	6.55
BO – 10	Beef ribs	10.44
BO – 11	Beef vertebra	7.69
BO – 14	Meat lunch + 12% bone	0.635
–	Bone meal	56.4

sodium fluoride. Thirty-six rats of about 50 gm in body weight were divided into 6 groups of 6 rats each. These groups were matched as to sex and litter. A sufficiently large batch of basal diet to last the duration of the experiment was made up and divided into 6 portions. To these portions was added sodium fluoride, mixed with casein for ease of incorporation in the diet. The basal diet was the stock room diet in use in this laboratory modified to contain about 0.22% calcium by analysis. It contained whole ground wheat 62%, crude casein 14%, whole milk powder 9.3%, wheat germ 5.5%, sodium chloride 1.0%, salt-free butter 6.1%, cod liver oil 2.0%, and calcium carbonate 0.1%.

TABLE 2

Retention of fluorine from sodium fluoride. (Mean values of 4 "runs" with maximum and minimum values)

AMOUNT OF FLUORINE ADDED TO BASAL DIET	AMOUNT OF ADDED FLUORINE CONSUMED	TOTAL CARCASS FLUORINE FOUND	CARCASS FLUORINE CORRECTED FOR BASAL	RETENTION OF ADDED FLUORINE
mg %	*mg/rat*	*mg/rat*	*mg/rat*	*%*
0.8	*5.13* 4.97–5.24	*2.43* 2.38–2.48	*2.17* 2.08–2.31	*42.3* 40.4–46.5
0.6	*3.60* 3.34–3.74	*1.83* 1.60–2.12	*1.57* 1.38–1.78	*43.6* 41.3–48.1
0.4	*2.42* 1.85–2.67	*1.35* 1.17–1.58	*1.09* 0.95–1.24	*45.0* 39.6–51.3
0.2	*1.23* 0.96–1.32	*0.86* 0.72–1.07	*0.60* 0.50–0.73	*51.6* 47.7–55.2
0.1	*0.57* 0.49–0.61	*0.60* 0.51–0.76	*0.34* 0.26–0.42	*59.8* 42.4–73.0
Basal diet	0.0	*0.26* 0.17–0.34	..	..

While this basal diet contained about 0.02 mg% of fluorine, this amount was small in comparison with the amounts added as supplements. Also one batch of diet was used for each "run," so that the fluorine in the basal diet was constant for all groups in that run, and a correction was easily made. The 6 diets used consisted of the basal diet alone and the basal diet plus, 0.1, 0.2, 0.4, 0.6 and 0.8 mg%, respectively, of fluorine. The 6 groups were fed these diets ad libitum for 8 weeks, records being kept of the amount of feed consumed. At the end of this time the rats were killed and the

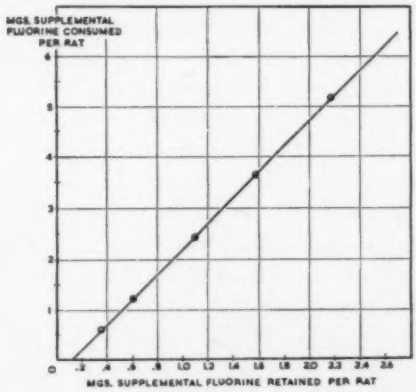

Fig. 1 The variation of fluorine retention with dietary sodium fluoride.

carcasses, less skin and gastrointestinal tracts, analyzed for fluorine.

Four "runs" were made in this fashion. Table 2 gives the mean values and the maximum and minimum values obtained. The retention of the added fluorine was over 40%, being greater at lower levels of fluorine. If the added fluorine retained (column 4) is plotted against the added fluorine consumed (column 2), a straight line is obtained. This is shown in figure 1. However, this curve does not pass through the origin of the axes but has an intercept corresponding to 0.12 mg retention at zero fluorine added.

Fluorine retention from bone

Experiment 3. This experiment was designed to compare the retention of fluorine when supplied as sodium fluoride with its retention when added as cooked bone in the food. As in experiment 2, 6 groups of 6 rats each were used in each "run." The basal diet was also the same as before. One or two groups of rats received the basal diet alone.[2] Another group in each run received the basal ration with 0.6 mg% fluorine added as sodium fluoride. The remaining groups each received rations containing 0.6 mg% fluorine as cooked bone. Each group received a ration supplemented with a different type of bone. To prepare these rations the amount of cooked bone required to add 0.6 mg of fluorine per 100 gm of dried final ration was calculated from the fluorine content of bone as given in table 1 and the moisture content. This amount of bone was then mixed with the required amount of basal ration. The mixture was spread on trays and dried with a current of air at about 40°C. The final total weight of the ration thus prepared was determined and the actual supplementary fluorine added was calculated from this weight and the known quantity of fluorine that had been added to the bone. This supplementary fluorine was thus not exactly 0.6 mg%, but varied from 0.55 to 0.65 mg%. The actual fluorine content was used to calculate the fluorine retention.

The diets were fed ad libitum for 8 weeks, a record being kept of the consumption of food. At the end of this time the rats were killed and the carcasses, less skin and gastrointestinal tracts, were assayed for fluorine. The percentage retentions were calculated in the same way as the results shown in table 2. These retentions were determined in triplicate for each type of bone tested, the triplicates always being done in different "runs" rather than together in one "run."

The results are given in table 3. The third column shows the amount of fluorine in the bone; these figures are taken from

[2] In early work one basal control and one with added fluorine at the 0.1 mg% level were included in each run, but later it was felt to be advisable to drop the 0.1 mg% level and include a duplicate control.

TABLE 3

Comparative retention by young rats of the fluorine of bone and of sodium fluoride when fed at a level of 0.6 mg of fluorine per 100 gm of diet

BONE SAMPLE NO.	TYPE	FLUORINE CONTENT OF BONE	RETENTION OF FLUORINE OF BONE *Mean* Min. – Max.	CONTROL RETENTION OF FLUORINE OF NA F. *Mean* Min. – Max.
		mg %	%	%
BO – 1	Regular run	4.97	*40.1* 36.0–45.1	*43.2* 42.4–44.6
BO – 4	Beef hard bones	14.97	*43.4* 39.6–45.4	*42.9* 42.4–43.8
BO – 5	Beef soft bones	21.5	*44.5* 41.9–47.1	*42.3* 42.0–42.5
BO – 6	Veal hard bones	1.24	*10.7* 7.6–15.5	*41.5* 40.0–43.8
BO – 7	Veal soft bones	1.16	*17.3* 16.4–18.7	*43.1* 40.0–44.6
BO – 8	Pork hard bones	7.39	*41.7* 35.8–47.4	*42.3* 40.0–46.0
BO – 9	Pork soft bones	6.55	*40.2* 35.6–43.4	*41.4* 38.5–43.8
BO – 14 [1]	Meat lunch + 12% bone	0.635	*38.0* 35.0–40.6	*42.8* 38.5–45.3
--	Bone meal	56.4	*34.2* 33.0–34.8	*45.3* 44.6–46.0

[1] This sample used at a level to add 0.3 mg % of fluorine to the basal diet.

table 1 and are shown here for easier comparison. Column 4 gives the mean retention observed for the fluorine of the bone, along with the maximum and minimum values obtained by the triplicate analyses. Column 5 shows the mean retention of the fluorine of the sodium fluoride in the same "runs." In the case of those bones containing the higher amounts of fluorine — that is, beef, pork, and possibly regular run — the observed retention was the same as that of the sodium fluoride controls. Since these bones contained large concentrations of fluorine, the amount of bone required to add 0.6 mg% of fluorine to the diet was small, so that the quantity of calcium added was also small. Thus the calcium in the diet with added beef soft bones was 0.367% compared to 0.22% in the basal diet, and the calcium in the diet with added regular run of bone was 0.695%.

On the other hand, the retention of fluorine added as veal bone was only one-quarter or one-third that of the sodium fluoride controls. In these cases the fluorine content of the bones was low, necessitating the addition of large amounts of bone to the basal diet to add 0.6 mg% of fluorine. Thus there was also an addition of considerable calcium, so that the diet containing veal hard bones had 1.82% calcium and that containing veal soft bones had 1.72% calcium. It is also interesting to note that the fluorine retention was significantly higher in the latter case despite the fact that, on the basis of the lower fluorine concentration in the bone only, one might expect that it would be lower. The low retention found with the bone meal cannot be explained on the basis of the calcium added to the diet. In this case the calcium concentration of the diet was only 0.252%, or lower than in any other diet with added bone. Therefore the fluorine must have been partially converted to a less available state during the processing of the bone meal.

Fluorine retention by adult rats

Experiment 4. Experiments 2 and 3 had used young growing rats, starting them at about 50 gm in weight and 6 to 8

weeks of age. These rats were in heavy positive calcium balance, retaining about 90% of the calcium in the basal diet and, presumably, large portions of the calcium in the bone mixtures. The large removal of calcium from the intestine might be expected to influence the fluorine retention. Experiment 4 was set up to investigate this point by using adult rats that had passed their period of rapid growth.

Rats were maintained on the normal stock room diet until 6 months old. At this time they were divided into 6 groups matched as to litter and sex. One group was killed and analyzed at once. The other 5 groups were placed on the basal diet, the diet with 0.6 mg% of fluorine added as sodium fluoride, and the diets with 0.6 mg% of fluorine added as beef soft bones, veal soft bones, and pork hard bones, respectively. As in the other experiments, they were fed these diets for 8 weeks, at which time they were killed and analyzed. Duplicate "runs" were made. The results are given in table 4, along with the retentions observed with the same products in the previous experiment. The retention of fluorine was considerably reduced in all cases, including that of sodium fluoride, from that found in the young rats. The retention from the beef (BO-5) and pork (BO-8) bones was lower than that of the sodium fluoride controls in this experiment, but still not markedly so. Once again, the retention from veal (BO-7) bones was considerably below that of the sodium fluoride controls.

Fluorine retention from drinking water

Experiment 5. In the experiments so far the sodium fluoride had been incorporated into the dry ration as a powder. No evidence of fluorosis or striations of the teeth (McClure, '39b) had been observed. It was possible that if sodium fluoride were given dissolved in drinking water, the topical application of the fluoride in solution might result in the teeth taking up more fluorine than by the ingestion of it in the food in a more or less insoluble form. The following experiment was designed to see: (1) if the concentration of fluorine in

TABLE 4

Fluorine retention in adult (6-month) rats. Comparative retention from bone and from sodium fluoride when fed at a level of 0.6 mg of fluorine per 100 gm of diet

FLUORINE ADDED AS:	ADDED FLUORINE CONSUMED PER RAT	FLUORINE FOUND IN CARCASS	FLUORINE FOUND CORRECTED FOR BASAL	RETENTION OF ADDED FLUORINE	MEAN RETENTION	COMPARATIVE RETENTION IN YOUNG RATS
	mg	*mg/rat*	*mg/rat*	%	%	%
Basal only	0	0.51 [1]				
	0	0.57 [1]				
Na F	3.44	1.64	1.13	32.8	30.5	42.7
	3.82	1.65	1.08	28.3		
BO – 5	3.76	1.72	1.21	32.2	27.7	44.5
	3.92	1.48	0.91	23.2		
BO – 7	3.82	1.09	0.58	15.2	14.5	17.3
	3.98	1.12	0.55	13.8		
BO – 8	3.70	1.59	1.08	29.2	25.6	41.7
	3.78	1.40	0.83	22.0		

[1] Adult controls contained 0.47 and 0.34 mg F. per rat at the beginning of the feeding period.

[2] See table 3, column 4.

the teeth bore a constant relation to that in the carcass as a whole whether the fluorine were given in the drinking water or as cooked bone in the food; and (2) if striations in the teeth could be produced at a lower total amount of ingested fluorine when the fluorine was given in the drinking water.

Forty-five rats of about 50 gm in weight were divided into 9 groups of 5 rats each. As it was not possible to match 9 groups for litter and sex, three of the groups were matched while the remaining 6 groups were matched in three pairs. One of the three matched groups was given the basal fluorine ration and distilled water to drink. This was the basal control group. The remaining two of these matched groups were given, respectively, 0.2 mg% fluorine as sodium fluoride in the drinking water and 0.4 mg% fluorine as pork soft bone in the feed. The remaining paired groups were given, similarly, 0.4 mg% fluorine in the water and 0.8 mg% in the feed; 0.8 mg% fluorine in the water and 1.6 mg% in the feed; and 1.6 mg% fluorine in the water and 3.2 mg% in the feed. In all cases those groups receiving the fluorine in the feed were given distilled water to drink, while those receiving fluorine added to the water were given the fluorine basal ration. The rats were maintained on this regime for 5 months, during which time complete records of food and water consumption were kept. At the end of this time the rats were killed. The incisor teeth, both upper and lower, were extracted, dried, and analyzed for fluorine. The remainder of the carcasses, less skin and gastrointestinal tracts, were also analyzed.

The results of the analyses are recorded in table 5. The first column gives the concentration of fluorine added to the drinking water and the feed. The next column shows the actual amount of supplemental fluorine consumed per rat. It can be seen that this amount for each group of a matched pair was comparable, i.e., the quantity of fluorine consumed by the group receiving 0.2 mg% in the water was about the same as that consumed by the group receiving 0.4 mg% in the ration, and so on. The fluorine concentration in the teeth, expressed as milligrams per gram of dry weight, is shown

TABLE 5

Comparative concentrations of fluorine in teeth and in the entire carcass after ingestion of fluorine in water or as bone

CONCENTRATION OF FLUORINE ADDED TO WATER OR FEED	FLUORINE CONSUMED PER RAT	FLUORINE CONCENTRATION IN TEETH	FLUORINE CONCENTRATION IN TEETH CORRECTED FOR BASAL	FLUORINE CONCENTRATION IN CARCASS	FLUORINE CONCENTRATION IN CARCASS CORRECTED FOR BASAL		RATIO FLUORINE IN TEETH, FLUORINE IN CARCASS
mg %	*mg*	*mg/gm*	*mg/gm*	*mg/gm*	*mg/gm*		
			Basal control				
0	0	0.012	0	0.0014	0		
			Fluorine added to drinking water as Na F				
0.2	8.52	0.1012	0.090	0.0109	0.0095		9.5
0.4	15.5	0.141	0.129	0.0212	0.0198		6.5
0.8	31.1	0.304	0.292	0.0380	0.0366		8.0
1.6	61.2	0.804	0.792	0.0740	0.0726		10.9
					Mean	=	8.7
			Fluorine added to feed as pork soft bone				
0.4	7.32	0.129	0.117	0.0178	0.0164		7.1
0.8	16.2	0.134	0.122	0.0157	0.0143		8.5
1.6	27.3	0.183	0.171	0.0225	0.0211		8.1
3.2	56.1	0.238	0.226	0.0269	0.0255		8.9
					Mean	=	8.2

in the third column, and this value corrected for that found in the basal controls appears in the 4th column. Similarly, the concentration of fluorine in the carcasses, expressed also as milligrams per gram of carcass, is shown in the 5th and 6th columns. The ratio of the concentration of fluorine in the teeth to that in the carcass is given in the last column. There is no apparent relation between this ratio and the concentration of the fluorine in the water or feed. The ratios found when the fluorine was added to the drinking water were rather erratic, these showing on the average 8.7 times the concentration of fluorine in the teeth as in the carcasses. The ratios obtained when fluorine was added to the feed as pork bone were more consistent; the average concentration of fluorine in the teeth was 8.2 times that in the carcasses. Since these mean ratios were practically identical, this experiment would indicate that there was no appreciable adsorption of fluorine by the teeth directly from the drinking water, and that the major portion, if not all, of the fluorine in the teeth was deposited there at the same relative rate as that at which it was deposited in the rest of the skeleton, whether it was administered dissolved in the drinking water or in a relatively insoluble form in the food.

Figure 2 presents another aspect of the data from this experiment. Here the total supplemental fluorine consumed is plotted against the fluorine retained in the rat carcasses. It may be seen that the retained fluorine is in a straight line relationship to consumption when the fluorine is administered in the drinking water. In fact, previous work in this laboratory has shown that when fluorine is given as sodium fluoride in the food, this straight line relation will hold up to a concentration of 20.0 mg of fluorine in 100 gm of diet. However, when the fluorine is given as cooked bone in the ration, the proportion retained falls off rapidly as the amount consumed increases. No doubt this is an effect of the increased concentration of calcium in the rations concurrent with the increased amount of fluorine when supplied as bone.

A careful examination of the rats' teeth was made immediately after sacrificing the animals. Fine striations were discernible in the teeth of two of the rats receiving 1.6 mg% of fluorine in the drinking water, and some striations in lesser degree in the teeth of one rat receiving 3.2 mg% of fluorine in the ration. No abnormalities were noticed in the rest of the animals. Thus there was too little evidence of toxicity to permit conclusive comparison of the relative toxic effects of the fluorine in the water and the food.

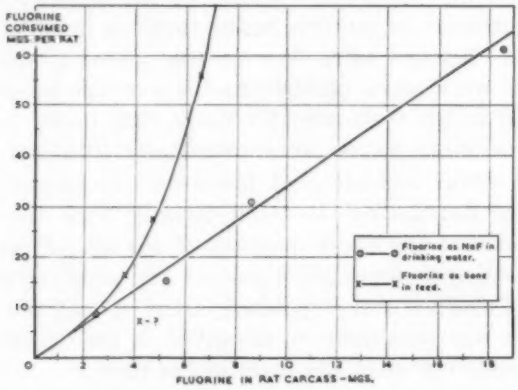

Fig. 2 The retention of fluorine from sodium fluoride in the drinking water and from bone added to the food.

Retention after long-term feeding

Experiment 6. The previous experiments involved feeding the fluorine supplements for relatively short periods of time. This experiment was set up to observe the effect of feeding a bone product for a long period. A commercial meat lunch preparation containing 12% added cooked bone was mixed with normal stock room diet in equal parts and was then air dried. This contained about 0.46 mg% of fluorine. A group of 24 young rats of about 50 gm in weight were fed this ration ad libitum. While no exact records of consumption were kept, the estimated fluorine ingestion was 0.08 mg

per rat per day. At the end of 6 months the rats were mated and a second generation was obtained and maintained on the same diet. Eight rats of the first generation were sacrificed after 6 months on the diet, 8 more after 12 months, and the remainder after 18 months. Similarly, 8 rats of the second generation were sacrificed at 6 months and the remainder at 12 months. The teeth were examined for fluorosis or striations and the carcasses analyzed for fluorine.

No striations or other evidence of toxic fluorine effects were observed. The rats became very fat on this diet and it was

TABLE 6

Effect of long term feeding of a bone product on the total fluorine in the rat carcass[1]

TIME ON DIET	MG FLUORINE RAT/CARCASS
1st generation — 6 months	3.22
1st generation — 12 months	4.25
1st generation — 18 months	3.05
2nd generation — 6 months	2.76
2nd generation — 12 months	3.08

[1] The diet was a 1:1 mixture of BO–14 and regular stock diet air dried. Fluorine concentration = 0.46 mg %. Estimated mean intake per rat per day = 0.08 mg.

difficult to breed them. It had been planned to carry a third generation on the diet but we were not able to produce this generation. Later experiments indicated that litters could be produced readily if the rats were bred at 4 months of age, and that there was no difference in ability to breed among rats on the normal diet, on the normal diet plus meat lunch with 12% added bone, and on the normal diet with meat lunch prepared without the added bone.

Table 6 gives the fluorine analyses of the rat carcasses. The total fluorine found per carcass was fairly constant and was independent of the length of time on the diet.

DISCUSSION

The observation that the level of fluorine in the rats' carcasses was the same whether the animals had been 6 months or a year and a half on the diet with added meat and bone gives rise to a theory that may explain some previously published divergent observations. This theory is that there is a concentration of fluorine in the skeleton that is indicative of the amount of dietary fluorine absorbed. When an animal is placed on a suddenly increased level of dietary fluorine there is, at first, considerable retention. As the concentration of fluorine in the skeleton builds up, there is a progressively increased rate of excretion of the fluorine, until finally the rate of excretion equals the rate of absorption and the concentration of fluorine in the skeleton becomes constant and remains so as long as the dietary level is maintained. Some evidence in support of this has already appeared in the literature. Lawrenz, Mitchell and Ruth ('40) fed levels of 3, 6 and 12 p.p.m. of fluorine as cryolite to rats for periods of up to 225 days. They did periodical balance studies during the feeding period and found greater excretion of fluorine the longer the time on the diets.

Machle, Scott and Largent ('42) found that a man ingesting 0.4 to 0.6 mg of fluorine per day was in non-cumulative fluorine balance. This was the amount that the man habitually consumed in his regular diet. When the intake was increased by the addition of 6.0 mg of fluorine daily as various test materials (Machle and Largent, '43), there was a retention ranging from 60% for sodium fluoride dissolved in the drinking water to 27.4% for bone meal mixed with the food. Retention was calculated as ingested fluorine minus the fecal and urinary excretion.

McClure, Mitchell, Hamilton and Kinser ('45) conducted extensive balance experiments on 5 young men. In these balance studies they included the loss of fluorine in sweat. They found that under "comfortable" conditions 77% of the absorbed fluorine was excreted by the kidneys and 24% in the sweat. Under "hot humid" conditions, 49% of the ab-

sorbed fluorine was excreted by the kidneys and 44% in the sweat. In either case they could find no cumulative fluorine retention when the test subjects were consuming as high as 6 mg of fluorine per subject per day. These workers conclude that fluorine ingested in food as bone meal, cryolite, or calcium fluoride in amounts up to 3.0 mg daily "may be expected, therefore, to not entail a hazard of cumulative toxic fluorosis." The positive retentions observed by Machle and Largent ('43) are at least partially explained by their failure to estimate excretion in the sweat. The subject of their balance study was returned to the normal level of fluorine ingestion after each test, so that there would have been little opportunity to build up an increased fluorine level in the body, and hence a positive retention would be expected. On the other hand, the subjects studied by McClure et al. were maintained on a continuous ingestion level of 6.0 mg per day for the entire study period, and the first complete balance studies were not made until after the subjects had been consuming this level of fluorine for 6 weeks. According to our theory, one would therefore expect at least a lowered retention if not complete balance under the latter conditions. This may then be an additional factor helping to explain the divergence in the observations made by the two groups of workers. Further investigations of this theory are being planned.

SUMMARY

Determinations have been made of the concentration of fluorine in various commercial classifications of bones.

Veal bones, both "hard" and "soft," had less than one-tenth as much fluorine as beef bones. Pork bones had an intermediate concentration.

Studies were made of the retention of fluorine from sodium fluoride, from cooked ground bone, and from bone meal.

The fluorine retained from sodium fluoride varied with the total fluorine ingested in a straight line relationship. The proportion of fluorine retained from added bone decreased as the amount ingested increased.

When fed at a level of 0.6 mg% in the diet, the retention of ingested fluorine was the same when added as either sodium fluoride or pork or beef cooked bone. The retention when added as veal bone was only one-fourth to one-third that of sodium fluoride. The retention when added as bone meal was less than when added as cooked ground bone.

Retentions of fluorine in adult rats were lower than in young growing rats but the relations stated above still held true. The concentration of fluorine in rats' incisor teeth bears a constant relation to its concentration in the rest of the carcass, and this relation does not change with changes in the amount of fluorine ingested, whether as sodium fluoride in the drinking water or as bone.

When rats were placed on a diet containing 50% of a meat product with added bone, the total fluorine in the rats' carcasses reached an amount within 6 months that remained constant for up to a year and a half in the first generation, and up to one year in the second generation, the experiment being concluded at this time.

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STUDIES OF DIETARY RESTRICTION AND REHABILITATION

I. WEIGHT CHANGES AND FOOD CONSUMPTION IN RATS [1]

ESTHER DA COSTA AND RUTH CLAYTON

Medical Nutrition Laboratory, Chicago, Illinois, an installation under the jurisdiction of the Surgeon General, Department of the Army

THREE FIGURES

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INTRODUCTION

The results of studies of dietary rehabilitation following food restrictions made on man have been reported by Berning ('49), Burger et al. ('45), Davidson et al. ('46), Drummond ('46), Hottinger et al. ('48), Keys ('46a, b), Metcoff and McQueeny ('46) and Stuart ('44), and studies on animals have been reported by Aron ('11, '14), Benditt et al. ('48), Frazier et al. ('47), Hoelzel and DaCosta ('33, '37), McCay and his associates ('42, '43, '45), Morgan et al. ('46), Quimby ('48), and Quimby et al. ('48). However, no experimental study of the effects of different types of rehabilitating diets following different types of food restriction seems to have been made heretofore. We therefore made a study on male albino rats to determine the effects of rehabilitating diets high in protein, fat or carbohydrate following restriction in the following forms: caloric restriction of an otherwise adequate diet, protein restriction with a modified carrot diet similar to that used by Cannon et al. ('44), and restriction to a synthetic vegetable-

[1] The opinions expressed in this paper are those of the authors, and do not necessarily represent the official views of any governmental agency.

like diet. The latter diet was used in the hope of producing nutritional edema with a diet of known composition.

Our object was to determine the effects of the different dietary regimens on body weight; food consumption; fat and water content of total carcass and major organs; specific gravity; plasma proteins; liver glycogens; the endocrines; hematocrit; hemoglobin; water exchange by means of sodium, potassium, and chloride determinations; spontaneous activity; food selection; and general behavior. Only the weight changes, food consumption, and caloric efficiency (calories consumed/gram gained) are being reported in this paper.

Three groups of 114 male rats (Sprague-Dawley and Penn. State) were used in this study. After a 14-day preliminary period on the control diet (table 1), they were fed either the control, low calorie, carrot, or synthetic vegetable-like diet for 93 days (13 weeks). The rats were weighed weekly.

The low calorie diet (table 1) was similar to the control diet, with added roughage, and was fed in amounts of 13 gm, or 51 cal., per rat per day at the beginning of the experiment; but to maintain a satisfactory weight decline, the amount of food fed daily was reduced to 7 gm during the final week of restriction. Thus the average amount fed was 9.9 gm, or 39.0 cal., per day. The carrot diet (carrot D) was a low protein diet and a modification of Cannon's carrot diet ('44). It contained an average of 1.8% protein and was fed at the rate of 30 gm, or 48.6 cal., per day. The artificial vegetable-like diet consisted of 5% protein, 10% inorganic salts, and 10% roughage and was fed in amounts similar to those of the low calorie diet. Thus, the average amount fed was 9.9 gm, or 37.7 cal., per rat per day. In addition, all rats were fed about 5 gm of lettuce weekly, which, with the 0.2 ml of oil supplement (table 1), added only about 1.5 cal. per rat per day to the caloric intake. At the end of 93 days, 6 rats from the control group and 8 from each of the three restricted groups were killed in order to determine the effects of food restriction.

The remaining restricted rats were rehabilitated by feeding them either the high protein, high fat, or high carbohydrate

diet (table 1) ad libitum. Food consumption was recorded daily. The rats were weighed daily during the first 4 days of rehabilitation but only once each week thereafter. Two rats fed each type of rehabilitation diet following each type of restriction were killed for analysis on days one, two, three and 4, and 4 controls from each type of rehabilitation diet were killed at the end of the 4th day of rehabilitation. Twenty-

TABLE 1

Diets used

DIETS	INGREDIENTS						CALORIC DISTRIBUTION			
	Casein (vitamin-test)	Fat	Glucose [1]	Carrots	Salt mixture	Rough-age [2]	Protein	Fat	CHO [3]	Calories
	%	%	%	%	%	%	%	%	%	*Cal./gm*
Control	20.0	8.0 [4]	68.0	0	4.0	0	18.9	17.0	64.1	4.2
L.C.[6]	18.2	7.2 [4]	61.8	0	3.6	9.1	18.6	18.1	63.3	3.9
C.D.[7]	0	3.1 [4]	26.1	69.3	1.5	0	2.0	17.7	80.3	1.6
S.V.S.[8]	4.5	7.2 [4]	70.0	0	9.1	9.1	4.6	17.8	77.7	3.6
H.P.[9]	60.0	8.0 [4]	28.0	0	4.0	0	56.6	17.0	26.4	4.2
H.F.[10]	30.2	52.4 [5]	10.8	0	6.6	0	18.8	74.7	52.4	6.4
H.CHO.[11]	14.4	3.8 [4]	78.0	0	3.8	0	13.0	8.6	78.4	4.0

Supplements

Vitamins added to 100 gm of all diets	*mg*
Thiamine hydrochloride	1.0
Pyridoxine hydrochloride	1.2
Riboflavin	1.6
Calcium pantothenate	5.0
Niacin	10.0
Choline chloride	400.0

Oil mixture fed weekly (1 or 2 drops/rat)	
tocopherol	5 mg
Halibut liver oil	4 ml
Corn oil	24 ml

[1] Cerelose.
[2] Fifty per cent gum acacia plus 50% Cellu flour.
[3] Carbohydrate.
[4] Wesson oil.
[5] Wesson oil 22.8%, plus Crisco 77.2%.
[6] Low calorie diet.
[7] Carrot diet.
[8] Synthetic vegetable substitute diet.
[9] High protein diet.
[10] High fat diet.
[11] High carbohydrate diet.

eight rats (4 controls and 8 from each type of rehabilitation diet) were killed for analysis after 42 and 95 days of rehabilitation.

RESULTS

The weight changes produced by the different types of food restriction and each type of dietary rehabilitation are shown in figures 1, 2 and 3. The greatest loss in weight during the restriction was sustained by the rats fed the carrot diet and

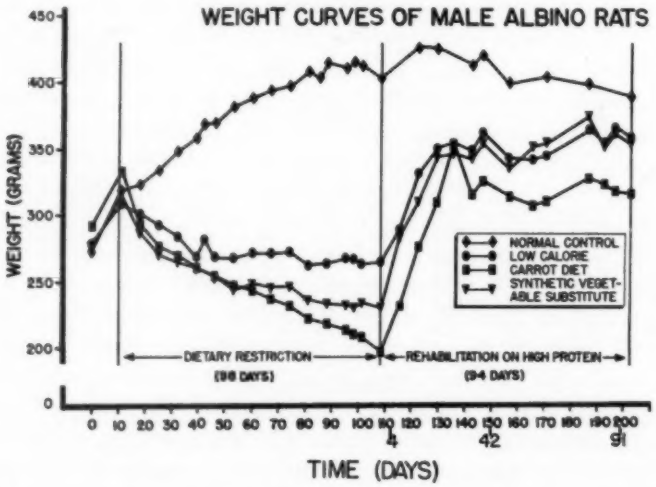

Fig. 1 Weight curves of male albino rats during 98 days of dietary restriction followed by 94 days of rehabilitation on a high protein diet (7/20/47 to 1/28/48).

the least by those fed the low calorie diet. During rehabilitation the greatest increases in weight with each type of rehabilitating diet occurred during the first 4 days, the high fat diet producing the greatest gains. Thus the weight of the rats fed the high fat diet following the low calorie diet (fig. 2) almost reached that of the controls. At the end of 42 days of rehabilitation the rats fed the high fat ration, following either the low calorie or synthetic vegetable diet, reached the weight level of the controls, and after 95 days of rehabilitation both

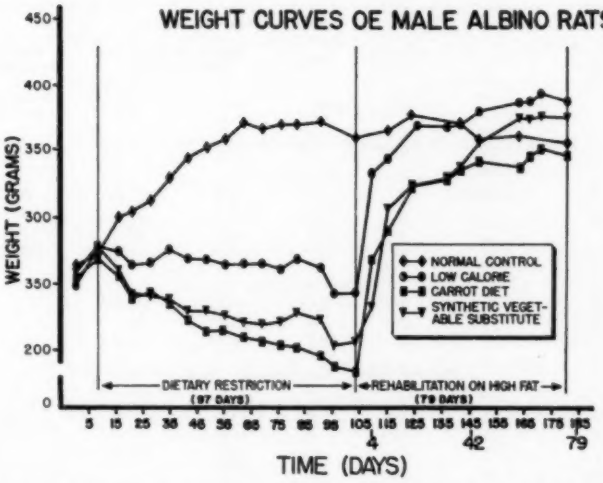

Fig. 2 Weight curves of male albino rats during 97 days of dietary restriction followed by 79 days of rehabilitation on a high fat diet (8/15/47 to 2/7/48).

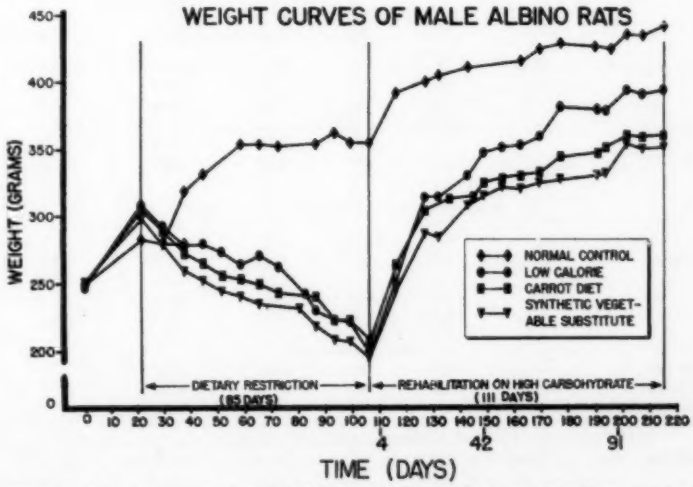

Fig. 3 Weight curves of male albino rats during 85 days of dietary restriction followed by 111 days of rehabilitation on a high carbohydrate diet (9/10/47 to 3/15/48).

TABLE 2

Food consumption and caloric efficiency during dietary restriction and re-feeding

DIETARY REGIMEN		RESTRICTION RE-FEEDING				
		Animals killed [1]	Time	Food consumption (Ave./rat/day)		Caloric efficiency
		no.	*days*	*gm*	*Cal.*	*Cal./gm gain in weight*
A. Rehabilitation on high protein diet (H.P.)						
			Controls			
Control	Control	6	98	18.4	77.8	96.5
		4	102	22.9	97.2	96.0
		4	140	11.2	47.5	119.2
		4	192	15.5	65.9	593.0
			Restriction period			
Low Cal.[2]	....	8	98	9.3	36.6 [3]	....
Carrot D.[2]	....	8	98	27.9	45.1 [3]	....
Syn. veg. sub.[2]	....	8	98	9.7	37.2 [3]	....
			Rehabilitation (high protein) (H.P.)[4]			
Low Cal.	H.P.[5]	2	1	37.0	157.0 [3,6]	7.9 [3,6,7]
		2	2	21.5	91.0 [6]	6.2
		2	3	17.0	72.0 [6]	13.3
		2	4	17.6	74.6 [6]	7.9 [3,7]
		8	42	18.1	77.0 [6]	26.1 [3,7]
		8	94	14.0	59.6	61.4
Carrot D.	H.P.	2	1	22.0	93.2 [3,6]	3.1 [3,6,7]
		2	2	14.3	60.6 [6]	3.6
		2	3	10.1	42.7	4.2
		2	4	11.3	48.1 [3]	3.4 [3,7]
		8	42	17.0	71.9 [3]	24.0
		6	94	15.2	64.7	41.5 [3]
Syn. veg. sub.	H.P.	2	1	32.0	135.5 [3,6]	5.4 [3,6,7]
		2	2	18.5	78.3 [6]	3.5
		2	3	19.1	80.6 [6]	6.0
		2	4	17.5	74.4 [6]	63.7
		8	42	14.8	62.8 [3]	25.6
		6	94	13.6	57.6	51.9
B. Rehabilitation on high fat diet (H.F.)						
			Controls			
Control	Control	6	97	21.4	90.8	94.3
		4	101	21.3	90.3	95.5
		4	139	20.7	87.9	90.0
		4	176	16.7	70.8	278.0
			Restriction period			
Low Cal.	....	8	97	10.0	39.5 [3]	....
Carrot D.	....	8	97	27.9	45.2 [3]	....
Syn. veg. sub.	....	8	97	9.54	36.5 [3]	....
			Rehabilitation (high fat) (H.F.)[4]			
Low Cal.	H.F.	2	1	25.5	163.0 [3,6]	15.3 [3,6,7]
		2	2	21.0	134.2	6.4
		2	3	23.0	147.0	7.6
		2	4	20.8	132.8 [3,6]	7.8 [3,7]
		7	42	15.1	96.5 [6]	25.3
		7	79	14.1	90.1 [3]	32.7 [3,7]

TABLE 2 (*continued*)

DIETARY REGIMEN		RESTRICTION RE-FEEDING				
		Animals killed [1]	Time	Food consumption (Ave./rat/day)		Caloric efficiency
		no.	*days*	*gm*	*Cal.*	*Cal./gm gain in weight*
Carrot D.	H.F.	2	1	7.0	44.7	8.2 [5,6,7]
		2	2	12.0	76.8	7.4
		2	3	9.3	59.5	5.2
		2	4	13.0	83.1 [5,6]	9.4 [5,7]
		8	42	13.2	84.6 [6]	22.7
		7	79	14.8	94.7	26.7 [5,7]
Syn. veg. sub.	H.F.	2	1	23.5	153.0	13.7 [5,6,7]
		2	2	20.0	128.0	6.7
		2	3	30.0	192.0	11.5
		2	4	20.6	131.9 [5,6]	8.4 [5,7]
		7	42	17.6	112.5 [5,6]	23.4
		8	79	15.6	99.5 [5]	26.7 [5,7]
C. Rehabilitation on high carbohydrate diet (H.CHO)						
			Controls			
Control	Control	6	85	20.8	88.0	88.2
		4	89	19.0	80.8	91.1
		4	127	18.3	77.5	83.0
		4	196	18.9	79.2	240.0
			Restriction period			
Low Cal.	....	8	85	8.8	34.9 [5]	....
Carrot D.	....	8	85	29.5	47.9 [5]	....
Syn. veg. sub.	....	8	85	9.2	35.4 [5]	....
		Rehabilitation (high carbohydrate diet) (H.CHO) [4]				
Low Cal.	H.CHO	2	1	20.0	80.8 [6]	2.0 [5,6,7]
		2	2	19.8	80.7 [6]	4.4
		2	3	34.0	138.5 [5,6]	5.1
		2	4	20.2	81.6 [6]	5.8 [5,7]
		8	42	19.4	78.6 [6]	16.6
		8	111	18.5	74.8 [6]	36.8 [5,7]
Carrot D.	H.CHO	2	1	28.5	116.2 [5,6]	10.0 [5,6,7]
		2	2	22.8	92.9 [6]	6.0
		2	3	24.1	98.6 [5,6]	8.9
		2	4	19.5	78.8 [6]	8.0 [5,7]
		8	42	18.5	74.7 [6]	10.7
		7	111	17.0	68.9 [6]	43.6 [5,7]
Syn. veg. sub.	H.CHO	2	1	13.5	50	1.7 [5,6,7]
		2	2	24.0	97.7	5.2
		2	3	15.5	63.1	4.5
		2	4	17.5	70.8 [6]	6.0 [5,7]
		8	42	18.4	74.4 [6]	9.7
		7	111	16.5	66.4 [5,6]	33.4 [5,7]

[1] Where less than 8 animals were sacrificed after 42 or 91 days of re-feeding, it means that the difference (one to two animals) had died during restriction.

[2] Low Cal., low calorie diet; Carrot D., carrot diet; Syn. veg. sub., synthetic vegetable diet.

[3] By Fisher's "t" test significantly different from control (p is less than 0.05).

[4] Mean age at beginning of rehabilitation, 24 weeks.

[5] High protein diet.

[6] By Fisher's "t" test significantly different from restricted.

[7] By Fisher's "t" test significantly different from 24-week-old rats (p is less than 0.05).

of these groups exceeded the weight of the controls. Rats restricted on the carrot diet were, in general, the slowest to regain weight and did not reach the control level in 95 days, even when fed the high fat diet.

Food consumption (in grams and calories) and the caloric efficiency (calories consumed per gram gained) are indicated in table 2. In general, the animals fed the high fat diet ate more than the controls (in terms of grams or calories). The rats rehabilitated on the high protein or the high carbohydrate diet usually ate less than the controls. The difference in food consumption, rather than some other factor, may explain the greater weight gains found in rats rehabilitated on a high fat diet. However, regardless of the rehabilitating diet, all rats showed a greater caloric efficiency during the first 4 days of rehabilitation than their controls of similar age (24 to 25 weeks). The caloric efficiency of the rats on the first day of rehabilitation was not easily estimated, because part of the weight gains were evidently due to a filling of the digestive tract. Nevertheless, the high caloric efficiencies of the second, third, and 4th days of rehabilitation resembled the caloric efficiency of 5-week-old rats, in which 8.5 cal. (2 gm) of the control diet were found to produce a 1-gm gain in body weight. As rehabilitation continues, caloric efficiency decreases, i.e., more calories of all diets are required for similar weight gains. However, at the end of 95 days of rehabilitation, the group rehabilitated on the high protein diet showed the poorest caloric efficiency, while the group rehabilitated on the high fat diet showed the highest caloric efficiency.

DISCUSSION

The average weight loss of restricted rats (28%) was similar to the approximately 25% weight loss reported by Brown ('46), Cartwright and Wintrobe ('46), Davidson et al. ('46), Keys ('46a) and Leyton ('46) in "starved" and "semi-starved" human beings. The length of the period of food restriction (93 days) used in this study corresponds to 4 or 5 years in the life of man.

If recovery of weight is regarded as evidence of successful rehabilitation, the high fat diet is clearly the superior one. However, the high fat diet (35%) used in this study produced no greater weight gains, or greater caloric efficiency, than a diet with 28% fat which was used in a preliminary study. This tends to agree with the findings of Scheer et al. ('47a, b) that a diet containing more than 20% fat is of no special value for rehabilitating rats.

It has been shown by Harte et al. ('48), and confirmed by the present authors, that the voluntary intake of the normally fed white rat is greatest at 5 weeks of age and levels off afterward. The high caloric efficiency of our rats during early rehabilitation therefore suggests that food restriction had a partial rejuvenating after-effect. Kunde ('23) found that the basal metabolic rate of dogs and human subjects increased after fasting and regarded this as evidence of rejuvenation such as Child ('15) previously produced by starvation in flatworms. Complete body weight recovery was obtained in this study only in rats rehabilitated with the high fat diet, but in a preliminary experiment in which the food intake of the rats was restricted for a period of only 70 days, all rehabilitated rats had equaled, or exceeded, the controls in weight at the end of the 70 days. This indicates a relationship between the length of the period of restriction and the possibility of complete recovery. Similar findings were reported by Aron ('11) on dogs and by Jackson and Stewart ('20) and Quimby ('48) on rats.

The difference in weight levels attained with rehabilitation in the present study was partly related to the type of restriction. Thus, all animals rehabilitated after having been fed the low calorie, but otherwise normal, diet, equaled or even exceeded the controls in weight at the end of the 91-day rehabilitation period, but the rats rehabilitated after having been fed the carrot diet or the synthetic vegetable diet did not attain the weight levels of the controls by the end of the rehabilitation periods. The carrot diet provided less than 2% protein and the synthetic vegetable diet 4.5% protein, while

the low calorie diet provided 20% protein. The carrot diet may, therefore, have produced greater weight losses than the other two diets used during restriction due to its extremely low protein content, despite its being fed in larger caloric allowances. After 5 months (150 days) of restriction, Cannon et al. ('44) found losses in body weight of 37.6% in rats fed their carrot diet providing 2% protein. Thus, there is evidently a level of dietary protein below which restriction during a 91-day period is likely to make a satisfactory recovery impossible in rats. That prolonged caloric deficiency is not likely to prevent recovery when the diet supplies protein and other nutritive essentials in more adequate amounts than a carrot diet, is indicated by the studies of McCay et al. ('45). These workers found that rats restricted calorically for as long as 700 days attained nearly normal weights when subsequently fed ad libitum.

SUMMARY

The effect of food restriction and subsequent dietary rehabilitation was studied on 342 young adult male albino rats. Fifty-four served as controls, 72 for restriction only, and 216 for 95 days of rehabilitation following 93 days of food restriction.

The effects of three types of dietary restriction and rehabilitation were determined. The dietary restrictions consisted of a quantitative reduction of a normal diet and two qualitative restrictions, i.e., a modified carrot (low protein) and a synthetic vegetable-like diet (low in proteins and high in salts). The rehabilitation diets included a high protein, a high fat, and a high carbohydrate diet. These diets, which were fed ad libitum, were isocaloric. Data were secured concerning changes in weight, food consumption, and caloric efficiency.

1. The rats lost an average of 28% in weight as a result of the different types and amounts of food restriction, but more weight (38%) was lost on the carrot diet than on the other restriction diets although almost three times as many calories were fed in this ration.

2. Independent of the type of preceding dietary restriction, the weight of the rats rehabilitated with the high fat diet increased more rapidly than the weight of animals rehabilitated with other diets. The high protein diet produced the slowest weight recovery and the high carbohydrate diet had an intermediate effect. At the end of the 95-day rehabilitation period, the rats rehabilitated on the high fat ration exceeded the control rats in weight, but the rats fed the high protein or the high carbohydrate diet did not quite equal the controls in weight.

3. In terms of calories per gram of food the intake during the first week of rehabilitation in the rats on the high fat diet was greater than in the controls.

4. During dietary rehabilitation all groups of rats showed an improved caloric efficiency (gain in weight per calorie consumed) when compared with the controls. Their level of caloric efficiency was equal to that of much younger (5-week-old) animals. This high caloric efficiency decreased with the completion of rehabilitation, but it was still greater than the caloric efficiency of the controls at the end of the rehabilitation periods.

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THE EFFECT OF METHIONINE SUPPLEMENTATION OF A SOYBEAN OIL MEAL—PURIFIED RATION FOR GROWING PIGS

J. MILTON BELL,[1] HAROLD H. WILLIAMS, J. K. LOOSLI AND L. A. MAYNARD

Departments of Animal Husbandry and Biochemistry and Nutrition, Cornell University, Ithaca, N. Y.

TWO FIGURES

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In line with current progress in determining the amino acid contents of feedstuffs, an understanding of dietary amino acid requirements is a prerequisite to any attempt to place protein nutrition on an amino acid basis. A significant series of investigations has been reported by Rose involving the evaluation of the qualitative and quantitative amino acid requirements of the rat, and, more recently, studies on man and the dog have been conducted. This work has been reviewed by Sahyun ('46). The findings of Bauer and Berg ('43) indicated qualitative similarity between the mouse and the rat in regard to amino acid nutrition. Almquist ('47) summarized the requirements of the growing chick and stressed the importance of the relationship between growth rates and minimum levels of the indispensable amino acids. A search of the literature reveals no attempts to evaluate the amino acid requirements of the larger species of farm animals, although in the case of swine it has long been recognized that the problem of protein nutrition is one of amino acid adequacy.

[1] Present address: Department of Animal Husbandry, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

Largely because of the dependence of growing pigs on quality of protein, studies were undertaken to gain information on the amino acid requirements of young pigs from weaning to 100 lb. live weight. Soybean oil meal was selected as the source of protein in order to evaluate methionine requirements. Previous work (Hayward and Hafner, '41; Mitchell and Block, '46) indicated that methionine is the limiting amino acid in soybean protein. Consequently, supplementation of the latter with methionine could be expected to yield an animal response in terms of biological value or growth, if the protein level were not too high.

EXPERIMENTAL PROCEDURE

The nitrogen balance technique was adopted because it permitted the calculation of biological values and was the most applicable of a number of methods reviewed (Boas-Fixen, '34; Bell, '48b). Records of growth responses were also kept. Young pigs, 4 to 8 weeks of age, were selected because of their high growth potential.

Adjustable cylindrical cages (Bell, '48a) were used during collections and for most of the preliminary periods; otherwise individual, litter-free floor pens were provided.

Carmine was employed in order to demarcate the fecal collections. Once daily the entire feces were placed in a large glass container with dilute HCl (50 ml of 0.1 N HCl in 18 l of H_2O) and 10 to 20 ml of toluene. This strength of acid was sufficient to yield an acidity of pH 5 to 6 in the final mixture, which contained just enough solution to cover the feces. The feces were stored in a refrigerator after collection and at the completion of a test period they were made into a slurry in a Waring Blendor, weighed, and aliquoted for analysis. Urine was collected in glass bottles containing 10 ml each of HCl and toluene. The urine was weighed and aliquoted (1/20) daily at 8:30 A.M.

Macro-Kjeldahl nitrogen analyses were made on all urine and feces samples. After being mixed a few minutes in the

Waring Blendor most fecal samples could be pipetted and weighed directly for analysis. In some cases where homogeneity was in doubt, an equal weight of concentrated H_2SO_4 was added to 50 to 100 gm of slurry and the mixture was allowed to digest overnight or longer on a steam bath. Moisture, ether extract, and fiber determinations on samples of feed and feces were conducted where necessary to compound diets or to compute digestibility of substances other than nitrogen.

An attempt was made to compound a semi-purified ration adequate in all factors likely to be essential to the growing pig. Attention was paid to the total and digestible energy of all diets, and to the fat and fiber levels particularly. Preliminary experiments indicated that 10% of fiber [2] in the dry matter gave more satisfactory laxation than a diet lower in fiber. As a consequence of this, a mixture of the following general composition was evolved: dextrose, 25; fiber, 10; salt mixture, 5; fat, 6; with starch, protein and vitamins comprising the remainder to make up 100 parts of diet.

Immediately before feeding, each allotment of feed was mixed with water in order to minimize wastage. A half-hour was usually sufficient time to allow for eating, after which the feed troughs were removed for collection of orts and for cleaning.

To study the adequacy of soybean protein for pigs, 4 diets were compared. Whole egg protein, known to have a high biological value (Mitchell and Carman, '24; Sumner, '38), served as a positive control. The protein of the other three diets was furnished by: (1) expeller processed soybean oil meal; (2) soybean oil meal plus methionine to equal the amount in the whole egg diet, and (3) soybean oil meal plus whole egg to replace 10% of the soybean oil meal protein, plus methionine. These diets were selected in order to determine whether soybean protein was equal to egg protein and, if not, whether methionine would completely supplement soybean protein, or whether some additional factor(s) from an

[2] Ruffex.

TABLE 1

Composition of diets

INGREDIENTS [1]		PROTEIN SOURCE			
		Whole egg	Soybean	Soybean + egg + methionine	Soybean + methionine
Soybean oil meal	(lb.)	....	21.36	19.00	21.12
Whole egg, dried extracted [2]	(lb.)	13.23	....	1.32	....
Corn sugar [3]	(lb.)	25.00	25.00	25.00	25.00
Starch	(lb.)	38.94	31.94	32.66	32.07
Corn oil	(lb.)	6.83	7.15	7.12	7.15
Ruffex (fiber) [4]	(lb.)	10.00	8.55	8.71	8.57
Salt mixture [5]	(lb.)	6.00	6.00	6.00	6.00
DL-Methionine	(gm)	....	....	81.82	87.40
Analysis of complete ration:					
Nitrogen content	(%)	1.68	1.69	1.68	1.69
Methionine content [6]	(%)	0.27	0.07	0.27	0.27
Calories/gm		4.41	4.39	4.43	4.38

[1] The following vitamins were added to each 100 lb. of ration: Thiamine hydrochloride, 93 mg; riboflavin, 140 mg; niacin, 466 mg; calcium pantothenate, 670 mg; pyridoxine hydrochloride, 106 mg; choline chloride, 45 gm; folic acid supplement (Folvite), 25 mg; alpha-tocopherol, 3 gm; vitamin A, 88,000 I.U.; and vitamin D, 35,200 I.U.

[2] Two sources of whole egg were employed in this study. Infertile incubator eggs — boiled, alcohol-ether extracted and dried — were fed during the first three periods of each replicate. The true digestibility was 82.2% and the B. V. was 86.5. In the last period it became necessary to substitute a commercial dried egg powder, similarly extracted; this yielded a true digestibility of 98.5% and a B. V. of 84.8. The low digestibility of the former product may reflect heat damage to the protein during drying, yet the B. V. was not inferior to that of the commercial preparation. It thus seems evident that the lower over-all efficiency of whole egg protein is chiefly, if not entirely, attributable to the differences in the digestible protein fed during the first three periods.

[3] "Cerelose," obtained from the Corn Products Co., Syracuse, N. Y.

[4] A rice hull preparation, Fischer Scientific Co.

[5] The following minerals were included in each 100 lb. of ration: NaCl, 8.542 kg; $CaHPO_4 \cdot 2H_2O$, 33.178 kg; KCL, 2.815 kg; MgO, 707.82 gm; $FeSO_4 \cdot 7H_2O$, 139.67 gm; KI, 9.38 gm; $MnSO_4 \cdot 4H_2O$, 8.65 gm; $CuSO_4 \cdot 5H_2O$, 8.35 gm; $CoCl_2$, 4.69 gm; and $ZnCl_2$, 4.44 gm.

[6] The methionine content of the diets was determined by microbiological assay. Hydrolysis with 10% HCl was carried out in the autoclave in sealed tubes and *Leuconostoc mesenteroides* P-60 # was used for the assay.

animal source would enhance the response to soybean protein. The diets were made to contain approximately 1.6% nitrogen (10% protein, N × 6.25). Feed was allowed at the rate of 50 gm per kilogram of live weight daily.

Two groups of 4 litter-mate Yorkshire barrows, selected for uniformity and thriftiness, were allotted to the 4 diets and studied in a 4 × 4 Latin square design. Nitrogen balance studies were carried out as the primary measure of the relative value of the proteins fed. The pigs were fed the diet under study for 20 days in a preliminary period, which was followed by two successive 4-day collection periods. Feed intake and growth data were also obtained during 20-day periods, including the collection periods, as a further measure of the responses to the dietary regimes. The diets fed are shown in table 1.

RESULTS AND DISCUSSION

Near the termination of the experiment two pigs died suddenly. Autopsies revealed some interesting conditions but no final diagnosis was made. When first examined several hours after death, the pigs were severely bloated, edematous in the inner thigh in the scrotum region, and had meningeal hemorrhages. No pathogenic organisms were identified in the tissues. Examination of the digestive tract revealed the evening meal still in the stomach along with much gas and a pronounced odor of fermentation by yeast. Yeast organisms among others, were present and active.

Following the final collections, the remaining pigs were slaughtered after feeding and examined at intervals. The hearts, lungs and digestive tracts, as well as the red cell counts and hematocrits, appeared to be normal, but the livers were about 50% larger than the "normal" values reported by Zeigler ('44). Alcohol analysis by the method of Newman and Abramson ('42) suggested the presence of alcohol in the blood, although well below intoxication levels for humans. While the cause of death was not ascertained, it appears possible that the large amount of sugar may have been a con-

tributing factor (Bachmann et al., '38) or that the combination of carbohydrates and cellulose may have resulted in excessive fermentation and gas formation, as observed by Woodman and Evans ('47).

Inasmuch as it was impossible to attribute the cause of death with certainty to any of the 4 diets being fed, and since only two pigs out of 20 maintained over prolonged periods on basically similiar diets were thus affected, analysis of the data was completed with due regard to the missing values (Snedecor, '46).

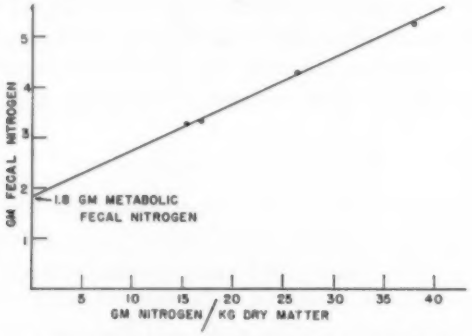

Fig. 1 The relationship between nitrogen intake and fecal nitrogen excretion.

In order to calculate the biological values of the diets fed it was necessary to obtain values for endogenous and metabolic nitrogen. The design of the experiment precluded the possibility of making these measurements on the same animals used in the balance and growth studies, because the pigs were started on balance trials as soon after weaning as they would eat the diets and were too large for further use at the close of the 4th period. Therefore other pigs of the same breed and age were used to measure the metabolic fecal nitrogen values for these diets. For this purpose the pigs were rotated according to a 3×3 Latin square design, so that each animal received each of three diets which contained 8.7%, 15.0%, and 21.7%, respectively, of protein supplied by

soybean oil meal. Two replicates were made, giving 6 observations on each protein level. Nitrogen balances and analyses of the excreta were carried out as described above. From the data obtained it was possible to find graphically by the method of Titus ('27) the fecal metabolic nitrogen (fig. 1).

In this figure it may be seen that approximately 1.8 gm of metabolic fecal nitrogen is excreted per kilogram of dry matter consumed. Since this excretion is mainly a function of the feed, this value would appear to be useful while the diets are kept basically the same (Mitchell, '34; Bosshardt and Barnes, '46).

TABLE 2

The weight of the pigs, the rate and efficiency of gain, the nitrogen retention and calculated biological value of the proteins

AVERAGE [1]	PROTEIN SOURCE			
	Whole egg	Soybean oil meal	Soybean oil meal + egg + methionine	Soybean oil meal + methionine
Body weight (lb)	76.8	77.1	77.2	76.5
Daily gain (lb.)	1.15	1.09	1.17	1.14
Feed per pound gain (lb.)	2.37	2.49	2.29	2.28
Digestible energy per pound gain (Therms)[2]	4.06	4.29	3.99	3.93
Apparently absorbed nitrogen retained (%)	72.3	66.5 [3]	74.0	73.3
Biological value	86.0	79.8 [3]	86.6	85.6

[1] Average values for two observation periods on each of 8 pigs.

[2] As determined using the bomb calorimeter.

[3] Statistically significantly lower than other values at odds of 99:1.

It is difficult to obtain a reliable value for endogenous urinary nitrogen in the growing pig. While it is theoretically desirable to make actual determinations, after consideration of the various difficulties involved and the wide inaccuracies in attempted measurements, the authors decided to use an estimated endogenous nitrogen value to calculate the biological values of the diets. Brody et al. ('34) derived the general equation $N_{mg} = 146\ W_{kg}^{0.72}$ as being applicable to mature swine. Treichler ('39), using growing rats, studied the relationship between endogenous nitrogen and basal calories. It

was suggested that the formula obtained would be applicable to other species by using Brody's ('27, '45) age equivalence diagrams. When Treichler's method was used in the present experiments with the formula $Y_{cal./day} = 73.4\ X_{kg}^{0.80}$ (Brody and Kibler, '44) to calculate the "resting" calories, the biological values were virtually identical in the case of small pigs with those obtained using Brody's equation, with a maximum increase of 2% at higher live weights. The pertinent data obtained from the 8 pigs are summarized in table 2.

It may be seen that on the unsupplemented soybean oil meal diet the pigs retained a significantly smaller proportion of the apparently absorbed nitrogen than on the other

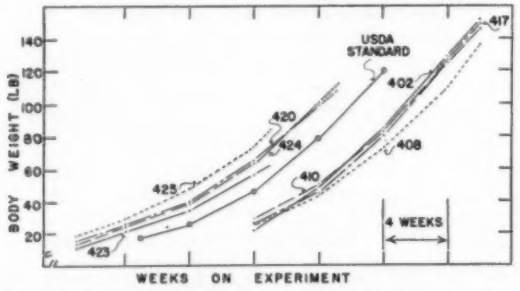

Fig. 2 Individual growth curves of the 8 pigs in comparison with the U. S. D. A. Standard.

diets, and that the calculated biological value of the soybean protein was significantly lower, accordingly. The rates of gain and the efficiency of feed and energy utilization were also lowest on the soybean oil meal diet, thus supporting the nitrogen balance data; however for these measurements the differences were not statistically significant because of the small number of animals studied and the short growth periods employed. The rates of gain in body weight made by the pigs during the study (fig. 2) were approximately equal to the U. S. Department of Agriculture standard for pigs of this size (Ellis and Zeller, '39).

Any change in feed utilization efficiency raises the question of the constancy of the composition of the gains produced.

The net nitrogen retained per pound of gain (truly digestible nitrogen minus urinary nitrogen) should reflect any major change from protein to fat deposition or vice versa. With the value for the soybean diet at 13.76 and that for the supplemented soybean diet at 13.75 gm nitrogen retained per pound of gain, there is indication that the gains were similar in composition.

These studies have demonstrated that the amino acid, methionine, is a dietary requirement for growing pigs. Soybean oil meal, when fed at a level to supply 10% of protein in the diet, is deficient in methionine for this animal. The addition of this amino acid to the soybean oil meal diet to increase the total methionine content of the diet to 0.27% resulted in growth efficiency and biological values equal to the values obtained from a diet containing whole egg protein. It is suggested on the basis of these studies that the methionine requirement of growing pigs is between 0.07 and 0.27% of the diet, when the diet contains 10% of protein.

SUMMARY

Studies were made of the effects of protein from whole egg and from soybean oil meal on the nitrogen retention and growth of pigs. When fed at a 10% level of protein the protein from soybean oil was less efficiently retained by growing pigs and had a significantly lower biological value than whole egg protein, but the addition of methionine to equal the amount in the whole egg made the soybean oil meal protein equal to the egg protein.

The inclusion of a small percentage of whole egg protein in the methionine-supplemented soybean oil meal diet did not further enhance the value of the protein.

On the semi-purified diet used in these tests, 1.8 gm of metabolic fecal nitrogen was excreted per kilogram of dry matter consumed.

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VITAMIN A UTILIZATION STUDIES

III. THE UTILIZATION OF VITAMIN A ALCOHOL, VITAMIN A ACETATE AND VITAMIN A NATURAL ESTERS BY HUMANS

ERLING F. WEEK AND FRANK J. SEVIGNE

Research Laboratory, Collett-Week-Nibecker, Inc., South San Franciso, California

TWO FIGURES

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Previous vitamin A utilization studies (Week and Sevigne, '49a, b) performed with chicks and rats have shown that, in addition to the stability and absorption per se factors described by Gray, Morgareidge and Cawley ('40), Clausen ('43), Hickman et al. ('44), Popper and Volk ('44) and others as affecting the relative biological response to differing chemical forms of vitamin A, factors influencing the hydrolysis *in vivo* of vitamin A esters may be of equal or greater importance.

The purpose of the present investigation was to determine the significance of these factors when considering vitamin A utilization by human subjects at high vitamin A intake levels.

It has been demonstrated that for humans the serum vitamin A level remains nearly constant during the day and on consecutive days under normal physiological conditions and with average nutrition (Popper and Steigmann, '43). The serum vitamin A level rises temporarily after ingestion of high doses of vitamin A and returns to approximately the normal level after 24 hours (Breese and McCoord, '40; Ralli, Bauman and Roberts, '41; Adlersberg and Sobotka, '43; Popper, Steigmann and Zevin, '43). This temporary rise and ensuing drop has been designated the tolerance curve, and may be assumed to indicate the efficiency of intestinal absorption of vitamin A

as demonstrated by Popper, Steigmann and Zevin ('43) and Steigmann and Popper ('44). These investigators have shown that the increase of the serum vitamin A level to its maximum concentration in the tolerance curve is not determined by the vitamin A concentration of the liver but is dependent chiefly on factors affecting absorption of the vitamin from the intestine. Under normal physiological conditions, vitamin A esters are hydrolyzed prior to absorption (Gray, Morgareidge and Cawley, '40; Clausen, '43; Popper and Volk, '44). In the present investigation the tolerance curve is assumed to reflect the combination of factors affecting vitamin A stability and hydrolysis *in vivo*.

TABLE 1

Physico-chemical characteristics of vitamin A concentrates

PHYSICO-CHEMICAL CHARACTERISTICS	VITAMIN A ALCOHOL	VITAMIN A ACETATE	VITAMIN A NATURAL ESTER 1	VITAMIN A NATURAL ESTER 2
$E^{1\%}_{1\ cm}$ 325 mμ	924	758	332	107
$E^{1\%}_{1\ cm}$ 325 mμ, corrected	860	710	305	102
$L^{1\%}_{1\ cm}$ 620 mμ	2200	1630	800	257
Vitamin A alcohol (%)	99.0	4.0	6.0	2.0
Vitamin A acetate (%)		96.0		
Vitamin A natural ester (%)	1.0		94.0	98.0

EXPERIMENTAL

The vitamin A alcohol and vitamin A acetate concentrates were prepared in the laboratory by procedures previously described (Week and Sevigne, '49a, b), using male soupfin shark liver oil as the source of vitamin A. Two natural ester preparations were used for the experiment. The first preparation (natural ester concentrate 1) was prepared in our own laboratory, using a procedure which does not involve the use of high vacuum distillation. The second preparation (natural ester concentrate 2) was a distilled vitamin A natural ester concentrate.

The spectrophotometric and chemical data relating to these concentrates are summarized in table 1.

The ultraviolet spectrophotometric measurements were determined on the unsaponifiable fractions of the respective concentrates in isopropanol, using a Beckman spectrophotometer. The $E^{1\%}_{1\,cm}$ 325 mμ values were corrected for the proportion of irrelevant absorption present in the region of the vitamin A absorption maximum according to the method described by Morton and Stubbs ('46). The $L^{1\%}_{1\,cm}$ 620 mμ values were measured on an Evelyn photoelectric colorimeter using the procedure of Dann and Evelyn ('38). The percentages of the alcohol and ester forms of vitamin A in the respective concentrates were determined by the procedures described by Reed et al. ('44).

The hydrogenated vegetable oil used for the investigation was a commercial brand of vitamin A-fortified (15,000 units per pound) margarine which, according to the manufacturer, contained the following ingredients: 80% vegetable oil, 15.8% skim milk, 3.6% salt, 0.4% monostearin, 0.1% vegetable lecithin and 0.1% benzoate of soda.

The human subjects used in these experiments consisted of 18 men and 7 women in normal health and not under medical treatment or on special diets.

Each supplement contained 134,000 μg of vitamin A ($E^{1\%}_{1\,cm}$ 325 mμ, corrected $\times$ 570) thoroughly blended into 50 gm of margarine. Except in those cases noted, 300 mg of mixed tocopherols were added to each vitamin A margarine preparation. The relatively small amount of vitamin A (15,000 units per pound) originally present in the margarine can be considered to have had no significant effects on the results of the experiment.

The amount of margarine fed was selected as being comparable to the normal amount of fat consumed by an average individual daily. In order to establish a reasonable basis for the tolerance curve, we chose to feed the total amount of margarine together with the respective vitamin A supplement at the breakfast meal. The fortified margarine preparation

TABLE 2

Serum vitamin A levels for 25 subjects after ingestion of vitamin A alcohol, acetate and two natural ester supplements, each containing 134,000 μg of the vitamin expressed as μg per 100 ml

SUBJECT AND SEX	AGE	WEIGHT	HEIGHT	ORDER OF FEEDING	TIME IN HOURS AFTER TAKING SUPPLEMENT						
					0	2	4	5	6	8	24
	years	lb.	in.				(*A*) *Vitamin A alcohol*				
1 ♂	30	175	71	CABD	34.8	429	938	1190	1080	713	91.6
2 ♂	29	155	70	BCAD	22.4	244	278	519	431	198	70.9
3 ♂	35	170	70	ABCD	43.0	485	1060	1300	1120	749	132.0
4 ♂	35	178	74	DCAB	37.5	515	1125	958	805	642	79.3
5 ♂	33	162	70	DABC	51.4	464	938	1170	995	820	82.3
6 ♂	42	129	63	DBCA	33.0	492	775	907	1010	840	75.1
7 ♂	29	133	66	DABC	62.1	639	721	758	526	339	112.0
8 ♂	25	165	67	AD	55.5	305	246	274	178	151	78.2
9 ♂	44	183	73	ACDB	47.7	203	655	499	270	226	81.1
10 ♂	21	170	74	ADBC	64.3	289	219	197	373	244	90.0
11 ♂	21	155	68	ADBC	70.9	514	321	437	512	244	102.9
12 ♂	28	180	68	ADBC	63.4	563	480	672	877	476	113.1
13 ♂	36	160	70	ADBC	68.3	547	778	499	294	272	118.0
14 ♂	32	190	71	AD	57.7	394	570	559	804	600	162.9
15 ♂	53	200	70	DACB	64.3	561	1234	1440	1423	999	162.9
16 ♂	21	150	70	DACB	51.1	283	415	472	291	300	85.4
17 ♂	32	150	69	DABC	55.7	339	488	549	441	437	108.0
18 ♂	32	150	71	DABC	77.1	390	1208	1093	815	360	98.6
				Average	53.3	425	692	750	680	478	102.5
19 ♀	44	112	61	DBCA	39.1	180	694	607	366	344	135.0
20 ♀	33	126	65	BACD	44.6	128	458	428	363	544	60.6
21 ♀	44	158	65	BACD	36.3	184	750	649	347	222	72.6
22 ♀	24	165	66	DBAC	45.7	317	133	205	297	215	61.4
23 ♀	22	145	67	DBAC	44.6	338	208	260	205	141	47.7
24 ♀	37	116	64	ADBC	40.3	398	689	591	449	334	56.9
25 ♀	31	124	68	ADBC	35.1	451	271	319	369	181	67.1
				Average	40.8	285	458	437	342	283	71.6
							(*B*) *Vitamin A acetate*				
1 ♂	30	175	71	CABD	34.0	245	601	781	940	659	91.6
2 ♂	29	155	70	BCAD	27.6	186	240	483	366	228	67.0
3 ♂	35	170	70	ABCD	52.0	168	490	763	799	874	128.6
4 ♂	35	178	74	DCAB	39.6	476	820	982	848	598	84.4
5 ♂	33	162	70	DABC	54.5	380	847	985	1120	862	79.6
6 ♂	42	129	63	DBCA	29.8	393	716	928	1170	886	80.4
7 ♂	29	133	66	DABC	57.6	458	530	630	580	280	71.2
8 ♂	25	165	67	AD	...	...	...	...	...	...	...
9 ♂	44	183	73	ACDB	60.6	313	583	547	265	223	89.7
10 ♂	21	170	74	ADBC	60.0	326	234	201	261	326	100.3
11 ♂	21	155	68	ADBC	70.0	261	482	549	411	282	94.3
12 ♂	28	180	68	ADBC	64.0	426	384	315	357	504	117.4
13 ♂	36	160	70	ADBC	68.0	461	714	540	315	219	103.4
14 ♂	32	190	71	AD	...	...	...	...	...	...	...
15 ♂	53	200	70	DACB	64.3	555	1315	1449	1133	681	154.1
16 ♂	21	150	70	DACB	51.1	287	248	155	122	102	84.6
17 ♂	32	150	69	DABC	55.7	66	129	441	742	483	77.1
18 ♂	32	150	71	DABC	78.0	261	707	776	626	507	97.7
				Average	54.2	329	565	658	628	482	98.3
19 ♀	44	112	61	DBCA	39.1	221	548	407	366	325	61.9
20 ♀	33	126	65	BACD	53.4	287	363	270	248	144	53.4
21 ♀	44	158	65	BACD	36.3	145	318	338	220	156	70.9
22 ♀	24	165	66	DBAC	37.1	175	205	150	251	181	61.4
23 ♀	22	145	67	DBAC	34.0	266	345	471	214	163	47.7
24 ♀	37	116	64	ADBC	40.0	535	475	522	324	197	74.0
25 ♀	31	124	68	ADBC	40.0	107	164	141	270	147	45.1
				Average	40.0	248	345	328	270	188	59.1

TABLE 2 (*continued*)

Serum vitamin A levels for 25 subjects after ingestion of vitamin A alcohol, acetate and two natural ester supplements, each containing 134,000 μg of the vitamin expressed as μg per 100 ml

SUBJECT AND SEX	AGE	WEIGHT	HEIGHT	ORDER OF FEEDING	TIME IN HOURS AFTER TAKING SUPPLEMENT						
					0	2	4	5	6	8	24
	years	*lb.*	*in.*				*(C) Vitamin A natural ester no. 1*				
1 ♂	30	175	71	CABD	39.0	154	285	406	425	320	69.1
2 ♂	29	155	70	BCAD	24.0	123	191	186	110	107	64.6
3 ♂	35	170	70	ABCD	52.9	294	632	718	718	447	78.1
4 ♂	35	178	74	DCAB	37.5	384	637	763	595	492	76.6
5 ♂	33	162	70	DABC	49.8	298	765	880	710	592	84.4
6 ♂	42	129	63	DBCA	34.5	311	811	1050	1170	649	68.6
7 ♂	29	133	66	DABC	43.9	394	544	585	403	367	93.9
8 ♂	25	165	67	AD	...	...	...	...	...	...	...
9 ♂	44	183	73	ACDB	47.7	73	253	373	416	535	81.1
10 ♂	21	170	74	ADBC	60.0	318	378	441	322	164	105.4
11 ♂	21	155	68	ADBC	70.0	274	657	579	411	195	98.6
12 ♂	28	180	68	ADBC	65.0	423	165	165	229	240	104.6
13 ♂	36	160	70	ADBC	60.0	362	649	598	277	255	93.1
14 ♂	32	190	71	AD	...	...	...	...	...	...	...
15 ♂	53	200	70	DACB	66.5	450	1166	1251	1183	927	184.3
16 ♂	21	150	70	DACB	50.0	137	283	334	300	403	81.1
17 ♂	32	150	69	DABC	57.0	103	382	501	715	450	79.6
18 ♂	32	150	71	DABC	64.2	205	411	702	803	390	93.0
				Average	51.4	269	513	596	549	408	91.0
19 ♀	44	112	61	DBCA	36.4	157	203	339	330	203	48.2
20 ♀	33	126	65	BACD	51.1	295	295	274	317	298	98.3
21 ♀	44	158	65	BACD	44.6	189	175	330	219	154	60.6
22 ♀	24	165	66	DBAC	47.7	150	167	154	183	270	70.0
23 ♀	22	145	67	DBAC	46.0	139	162	141	163	212	64.0
24 ♀	37	116	64	ADBC	40.0	321	689	599	377	351	55.1
25 ♀	31	124	68	ADBC	39.0	248	254	231	236	168	55.0
				Average	43.5	214	278	295	261	237	64.5
							(D) Vitamin A natural ester no. 2				
1 ♂	30	175	71	CABD	35.7	168	317	429	533	353	82.6
2 ♂	29	155	70	BCAD	28.5	150	209	218	114	87	73.6
3 ♂	35	170	70	ABCD	43.8	240	583	628	641	416	64.5
4 ♂	35	178	74	DCAB	36.5	333	607	805	691	526	82.6
5 ♂	33	162	70	DABC	46.5	393	870	931	817	643	79.6
6 ♂	42	129	63	DBCA	36.5	402	685	997	1230	895	82.6
7 ♂	29	133	66	DABC	68.5	388	539	539	298	267	84.8
8 ♂	25	165	67	AD	55.5	242	292	260	165	146	64.5
9 ♂	44	183	73	ACDB	64.0	214	583	535	380	227	81.1
10 ♂	21	170	74	ADBC	50.3	300	253	171	163	343	98.6
11 ♂	21	155	68	ADBC	70.9	300	242	345	270	441	105.4
12 ♂	28	180	68	ADBC	66.0	227	422	363	285	353	100.3
13 ♂	36	160	70	ADBC	58.9	399	666	418	315	390	105.1
14 ♂	32	190	71	AD	70.8	296	667	609	657	474	248.6
15 ♂	53	200	70	DACB	66.6	377	776	941	926	549	115.7
16 ♂	21	150	70	DACB	50.0	204	180	287	223	334	70.0
17 ♂	32	150	69	DABC	56.0	103	378	506	703	449	77.1
18 ♂	32	150	71	DABC	62.0	189	399	722	797	393	92.6
				Average	53.7	274	482	539	512	405	95.0
19 ♀	44	112	61	DBCA	40.0	195	507	362	271	189	84.5
20 ♀	33	126	65	BACD	44.6	240	472	510	240	219	98.3
21 ♀	44	158	65	BACD	37.1	282	604	484	235	187	60.6
22 ♀	24	165	66	DBAC	51.1	141	186	231	310	211	62.3
23 ♀	22	145	67	DBAC	29.7	182	197	214	212	244	57.7
24 ♀	37	116	64	ADBC	40.3	269	724	814	737	441	89.4
25 ♀	31	124	68	ADBC	39.0	237	240	226	249	177	50.0
				Average	40.3	221	419	406	322	238	71.8

was eaten with toast or bread and coffee, except that subjects 4, 5 and 6 received two eggs and two slices of lean bacon in addition. However, the breakfast for each subject was duplicated on each of his 4 test days.

Samples of blood were taken by venous puncture before feeding the vitamin A preparations and at 2, 4, 5, 6, 8 and 24 hours afterwards. The subjects received a different vitamin A concentrate preparation on each Monday and Thursday for two consecutive weeks, with the exception of subjects 12, 13, and 15 to 18, inclusive. The latter were fed the usual two vitamin A preparations during the first week of the test period, as described. However, due to conflicting time schedules, a period of one week elapsed before the final supplements could be taken to complete the series. Subjects 8 and 14 were tested on two chemical forms of vitamin A only, receiving the supplements on Monday and Thursday of the same week. No evidence was found to indicate that the aforementioned slight variations affected the course of the experiment.

After eating the breakfast meal, the subjects were free to pursue their normal daily activities, reporting only at the specified times to have blood samples taken. They were instructed to follow their usual diets but to attempt to eat the same foods on each respective test day. Abstinence from alcoholic beverages was specified during the test period.

A system of rotation in administering the respective vitamin A concentrates to the various subjects was employed in an attempt to minimize variations in the serum vitamin A levels, as shown by the tolerance curves, which might be attributed to the relative order in which the various preparations were fed, although the literature indicates that the level of liver stores should not affect the tolerance curve (Popper, Steigmann and Zevin, '43; Steigmann and Popper, '44). The order of rotation in feeding the vitamin A preparations to the individual subjects is shown in table 2.

The blood samples were analyzed in duplicate for vitamin A and carotene, using a procedure based on the techniques of Kimble ('39), Glover, Goodwin and Morton ('47a, b) and

Van Bruggen ('48). Vitamin A was estimated by the Carr-Price reaction, using an Evelyn colorimeter equipped with a 620 mμ filter and calibrated against the U.S.P. Vitamin A Reference Standard Oil. Vitamin A values reported in this investigation were corrected for the blue color contribution of the carotene present in the sample.

DISCUSSION OF RESULTS

Relationship between chemical form of vitamin A and tolerance curves

The individual data for the 25 subjects are summarized in table 2. Average serum vitamin A levels for the 18 men and 7 women are shown in figure 1.

The data contained in table 2 were subjected to statistical analyses employing a replicated comparison of the individuals as the simplest and most effective means for testing the observed differences in response between any two of the chemical forms of vitamin A administered. The student "t" test was used to evaluate the probability that the observed mean differences were due to chance. Two methods of approach were used in evaluating the statistical significance of the data. In the first, comparisons of the vitamin A serum levels were made at each indicated hourly interval, i.e., 2, 4, 5, 6, 8 and 24 hours. The second treatment involved a comparison of the total area under the individual tolerance curves for each chemical form of the vitamin A fed. The method used in calculating the area under the tolerance curve is illustrated in figure 2 and the area, in this case, can be said to represent the increase in the vitamin A serum level from the normal (zero hour) value, expressed in terms of microgram-hours.

Comparisons were made for both the zero- to 24-hour and the 2- to 8-hour periods. The total area under the tolerance curve for the latter interval appears to afford the most valid means of comparison, as the greater part of the absorption takes place during this period. The degree of significance obtained by this statistical treatment of the data is shown in

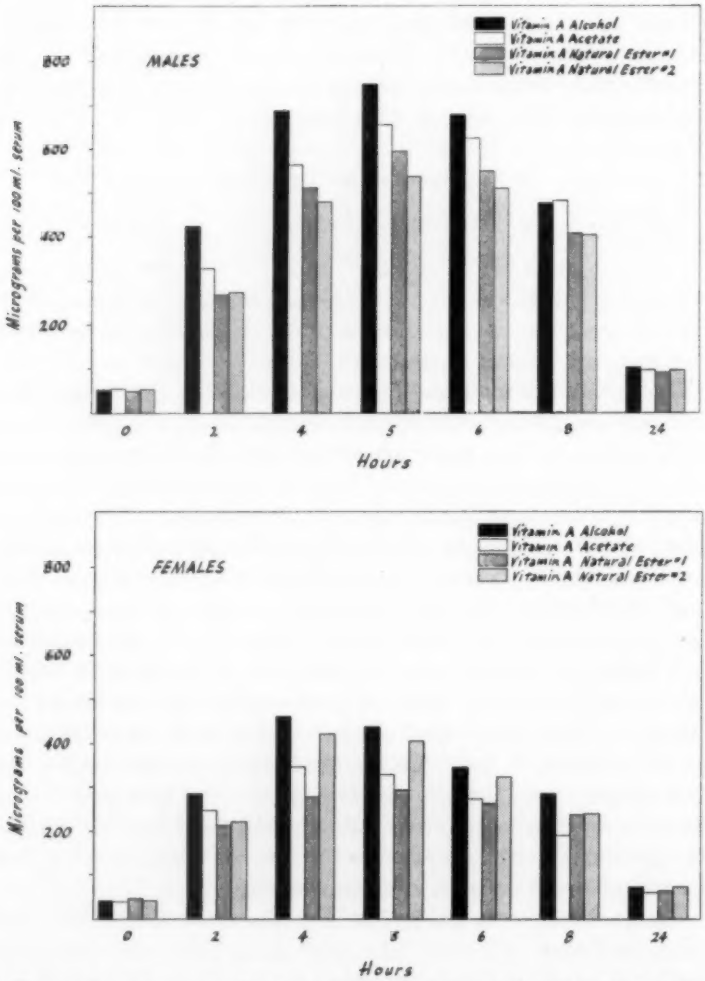

Fig. 1 Average serum vitamin A levels for male and female subjects before and after administration of supplements containing 134,000 μg of vitamin A.

table 3. It should be mentioned that other statistical treatments previously described produced approximately the same degree of significance as was obtained by using the area under the tolerance curve for the 2- to 8-hour period.

From the analysis shown in table 3 it may be seen that the chemical form of vitamin A fed has a significant effect on the absorption of the vitamin.

In the case of the male subjects, under the conditions of this experiment, vitamin A alcohol is very significantly superior to vitamin A acetate which, in turn, is significantly superior

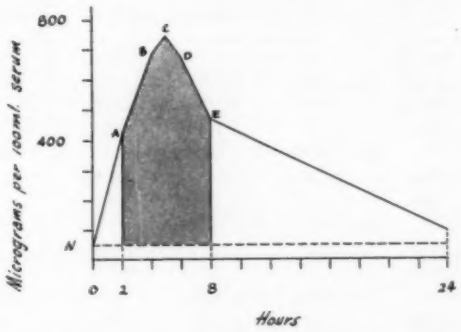

Fig. 2 A typical tolerance curve. Shaded area represents increase in vitamin A level for 2- to 8-hour period, expressed in microgram-hours. The calculated area = (A + C + E) + 1.5(B + D) − 6N.

to vitamin A natural esters. The two vitamin A natural ester concentrates fed did not demonstrate sgnificantly different responses. The maximum difference found in the male subjects was between vitamin A alcohol and vitamin A natural ester no. 2, where the probability of repeating the experiment with substantially the same result is shown to be better than 999 chances out of 1,000.

As the stability of vitamin A alcohol is known to be inferior to that of vitamin A esters under most conditions, it may be assumed that the relatively less favorable response to vitamin A esters is due to factors influencing the hydrolysis *in vivo* of these esters. The principal unfavorable factor in this case

is believed to be the concurrent feeding of a relatively large amount of fat (50 gm of margarine).

This group of women showed significantly ($P < 1\%$) lower utilization of the vitamin than the men for all vitamin A preparations administered. The women showed a significantly superior response to vitamin A alcohol compared to vitamin

TABLE 3

Statistical summary of data in table 2. Comparison of areas under the tolerance for 2- to 3-hour period

COMPARISON	MALES				FEMALES			
	M [1]	N [2]	"t"	"P"	M [1]	N [2]	"t"	"P"
Alcohol	3545				1960			
vs.		16	3.76	$P < 0.01$		7	2.45	$P = 0.05$
Acetate	2934				1448			
Alcohol	3545				1960			
vs.		16	3.66	$P < 0.01$		7	3.59	$P < 0.02$
Nat. ester 1	2558				1293			
Alcohol	3390				1960			
vs.		18	4.17	$P < 0.001$		7	1.23	Not sig.
Nat. ester 2	2385				1735			
Acetate	2934				1448			
vs.		16	2.14	$P = 0.05$		7	0.80	Not sig.
Nat. ester 1	2558				1293			
Acetate	2934				1448			
vs.		16	2.99	$P < 0.01$		7	1.22	Not sig.
Nat. ester 2	2436				1735			
Nat. ester 1	2558				1293			
vs.		16	0.85	Not sig.		7	3.31	$P < 0.02$
Nat. ester 2	2436				1735			

[1] M — mean of N observations expressed as microgram-hours.
[2] N — number of subjects taking both supplements.

A acetate and natural ester no. 1. The comparison of the alcohol form with natural ester no. 2 showed no significant difference, as contrasted to the highly significant relationship shown in the male group.

The women showed no significant differences in response between vitamin A acetate and either of the natural ester preparations. Furthermore, they reversed the order of utilization found in the male group for the natural ester prep-

arations to such a degree that natural ester no. 2 was shown to be significantly superior to natural ester no. 1 for the female group.

The differences in the levels of significance between the male and female groups for the comparisons shown in table 3 can be attributed, in part, to the smaller number of female subjects available for the experiment.

TABLE 4

Serum vitamin A levels for three male subjects fed supplements of 134,000 μg of vitamin A alcohol, with and without simultaneous ingestion of 300 mg of mixed tocopherols

TIME HOURS	SUBJECT NO. 4		SUBJECT NO. 5		SUBJECT NO. 6	
	Without tocopherols	With 300 mg tocopherols	Without tocopherols	With 300 mg tocopherols	Without tocopherols	With 300 mg tocopherols
0	34.5	37.5	44.5	51.4	34.2	33.0
2	545.0	515.0	447.0	464.0	474.0	492.0
4	1150.0	1125.0	875.0	938.0	756.0	775.0
5	970.0	958.0	1090.0	1170.0	888.0	907.0
6	790.0	805.0	983.0	995.0	990.0	1010.0
8	595.0	642.0	768.0	820.0	770.0	840.0
24	80.1	79.3	81.1	82.3	80.2	75.1

The authors believe that the differences in the relative utilization of the various forms of vitamin A by the two sexes may be connected with other variations in response which were noted.

Among the men it was usual to observe a rather smooth and regular rise and fall in the response curve. In 68 feedings to male subjects only 14 "delayed" response curves were noted. Such "delayed" curves showed double peaks, or a prolonged plateau at the top of the absorption curve. This type of tolerance curve probably paralleled delayed passage of a part of the fat and accompanying vitamin from the stomach to the small intestine.

In 28 feedings to women, 14 "delayed" response curves were noted. The postulated delay in the stomach would tend to cause relatively greater destruction of vitamin A prior to passage into the intestine.

Effect of added tocopherols on absorption of vitamin A

Several male subjects were fed vitamin A alcohol without tocopherols, as well as with tocopherols. Results are shown in table 4. Although the data are insufficient to establish significance, we do not believe that the simultaneous feeding of 300 mg of mixed tocopherols had an appreciable effect on vitamin A absorption.

TABLE 5

Free and esterified vitamin A in human blood

SUPPLEMENT	TIME AFTER ADMINISTRATION OF VITAMIN A (HOURS)							
	0		5		6		24	
	Micrograms of vitamin A per 100 ml of serum							
	Alcohol	Ester	Alcohol	Ester	Alcohol	Ester	Alcohol	Ester
Subject 7 (male)								
Vitamin A alcohol	54.6	7.5	60.6	697	52.6	473	54.8	57.2
Vitamin A acetate	51.8	5.9	50.0	580			49.8	21.4
Vitamin A ester no. 2	56.0	12.5	59.4	480			51.0	33.8
Subject 19 (female)								
Vitamin A alcohol	34.0	5.1	66.8	540	51.2	315	42.3	92.7
Vitamin A acetate	31.3	7.8	36.6	370			39.0	22.8
Vitamin A ester no. 2	34.6	5.4	26.7	335			33.0	52.4

Concentrations of the free and esterified forms of vitamin A in the blood

For subjects 7 and 19, the concentrations of the alcohol and ester forms of vitamin A present in the blood were determined at various time intervals during the test period. The chromatographic method described by Glover, Goodwin and Morton ('47a) was used to determine the concentrations of the two forms present. The results are shown in table 5.

Under normal conditions, the greater proportion of the vitamin A in human blood serum was present in the alcohol form.

However, after ingestion of 134,000 μg of the vitamin, the increased vitamin A in the blood serum was present as the ester, irrespective of the chemical form of the vitamin administered in the supplement.

SUMMARY

1. Blood serum vitamin A levels were used as the criterion for determining the relative utilization of the alcohol, acetate and natural ester forms of vitamin A by 18 men and 7 women, after administration of 134,000 μg of the vitamin contained in 50 gm of margarine.

2. For the male subjects, vitamin A alcohol showed greater biological efficacy than vitamin A acetate which, in turn, was superior to the natural ester forms. Vitamin A alcohol produced a greater biological response than vitamin A acetate for the female group; however, comparison of the alcohol form with a distilled natural ester preparation showed no significant difference in utilization.

3. The addition of 300 mg of tocopherols to the vitamin A alcohol supplements had no appreciable effect on vitamin A absorption.

4. After ingestion of high dosages of the vitamin, the increased vitamin A in the blood serum was present as the ester, irrespective of the chemical form of the vitamin administered in the supplement.

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PANTOTHENIC ACID DEFICIENCY AND CONJUGATION REACTIONS IN VIVO

II. THE EFFECT OF RIBOFLAVIN AND PYRIDOXINE DEFICIENCIES ON THE ACETYLATION OF SULFANILAMIDE[1]

MAURICE E. SHILS, SARAH ABRAMOWITZ AND MARTIN SASS

School of Public Health, Columbia University, New York, N. Y.

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Following the discovery that pantothenic acid is a constituent of coenzyme A (Lipmann et al., '47), it was demonstrated that pantothenic acid deficiency in rats decreased acetylation of aromatic amines *in vivo* (Riggs and Hegsted, '48; Shils, Seligman and Goldwater, '49). While the effect of pantothenic acid on acetylation rests on the solid foundation of its coenzyme function, it is conceivable that other vitamin deficiencies may influence the acetylation process through subsidiary effects, so that pantothenic acid deficiency would not be unique in this respect. Consequently, a survey of the effects on acetylation of deficiencies of other vitamins is worthwhile in order to establish the specificity of pantothenic acid in this connection.

Thiamine deficiency has been found not to influence the rate of acetylation of sulfanilamide which was administered at two different levels (Shils et al., '49). Riggs and Hegsted ('49) investigated the effects of thiamine deficiency and of riboflavin deficiency on the ability of rats to acetylate *p*-aminobenzoic acid (PABA) fed in various amounts. At the lowest and highest levels of PABA, thiamine deficiency had

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no effect, and at intermediate levels "the difference is not striking." Acetylation values for riboflavin-deficient rats were found to be significantly lower than those for the controls except at the highest level of PABA, while pantothenic acid deficiency produced the greatest reduction in acetylation. Martin and Rennebaum ('43) had reported that thiamine and riboflavin deficiencies decreased the proportion of acetylsulfanilamide in the blood of rats, whereas deficiencies of pantothenic acid and pyridoxine did not. The results of our studies on riboflavin and pyridoxine deficiencies in relation to acetylation are reported here.

METHODS

The control diet had the following percentage composition: casein [2] 20, cerelose 64.5, corn oil 10, salts (U.S.P. no. 2) 4, roughage [3] 1.5, plus the following vitamins added per 100 gm of basal diet: thiamine-HCl 1 mg, riboflavin 1 mg, pyridoxine-HCl 1 mg, niacin 2 mg, folic acid [4] 0.20 mg, biotin 0.002 mg, choline 100 mg, and percomorph oil 0.12 ml.

The riboflavin-deficient and pyridoxine-deficient diets had the same composition as the control diet, with omission of the respective vitamins.

Urine collections were made for periods of 22 hours, using individual rats in metabolism cages standing over large funnels, with a bulb below for separating urine and feces. In order to avoid the possibility of "contamination" of cages with vitamins during the urine collections, a vitamin-free diet was given during this 22-hour period. This consisted of casein 18, cerelose 69, corn oil 5, salts (U.S.P. no. 2) 4 and roughage [5] 4. A sulfanilamide solution (0.8% in water) was injected intraperitoneally as follows: 3 ml at the beginning of the urine collection period, 2 ml at the end of the first

[2] Labco, vitamin-free.

[3] Ruffex, a material prepared from rice hulls and obtained from Eimer and Amend, New York, N. Y.

[4] Folvite.

[5] See footnote 3.

hour, and 2 ml at the second hour, for a total of 56 mg of the aromatic amine. Free and total sulfanilamide were estimated colorimetrically by the method of Bratton and Marshall ('39). Acetylation rates are expressed as the percentage conjugation of the sulfanilamide excreted in the urine.

EXPERIMENTAL PROCEDURE AND RESULTS

Two series of experiments were performed, each comprising control, riboflavin-deficient and pyridoxine-deficient groups. In series I the control and deficient groups each consisted of 8 male litter-mate rats, initially averaging about 70 gm in weight. By the end of a month the deficient rats were exhibiting little or no growth and the control rats were then restricted in their food intake to approximately the amounts eaten by the deficient animals. This was done to help eliminate any effect on acetylation of variation in the quantity of food intake per se and to limit the weight of the controls. It was found in performing acetylation tests with the rats of series I that when the animals weighed less than approximately 100 gm the urinary acetylation test was often unsatisfactory on several counts, since either the excretion of sulfanilamide was markedly incomplete (i.e., less than half of that injected as compared to the usual range of 70 to 90%) or else the percentage of acetylation tended to be low, with spontaneous and sometimes marked increase without treatment as the animals became heavier. Series II was performed to substantiate the data of the first series, where one-half of the pyridoxine-deficient rats and one-fourth of the riboflavin-deficient animals had died before the repletion period. In an attempt to eliminate the erratic results experienced with some of the light-weight rats, the latter series was begun with somewhat older animals. Ten litter-mate male rats were placed on each of the riboflavin- and pyridoxine-deficient diets, with 5 rats acting as controls; their initial weights averaged about 95 gm. Even with the heavier starting weight, many of the riboflavin-deficient rats and a few of the pyridoxine-deficient animals fell below 100 gm in weight. The rats were

then given occasional injections of 20 μg of riboflavin or 30 μg of pyridoxine to boost weight slowly. No acetylation tests were performed on these supplemented animals until a decline in weight had developed again. Numerous tests of acetylation ability were made during the course of the deficiencies and several tests were carried out after replacement of the missing vitamin both by injection and by restoration in the diet. In series I 8 acetylation tests were performed; namely, on the 37th, 50th, 64th, 99th, and 113th days on the experimental diets and, following restoration of the missing vitamins, on the 120th, 129th and 137th days. In series II 5 acetylation tests were made with the riboflavin-deficient group before restoration of riboflavin and two tests afterwards (on the 30th, 58th, 69th, 92nd, 114th, 121st, and 133rd days); with the pyridoxine-deficient group, 6 tests were made during the deficiency period and three afterwards (on the 58th, 64th, 86th, 126th, 148th, and 154th, 155th, 161st, and 168th days).

The results obtained with series I were consistent among the animals of a given group; partial protocols of three of the rats surviving the deficiencies and of three control animals in this series are given in table 1; included are the weights of the animals at the time of the acetylation tests. At the bottom of this table are the group averages for acetylation of the surviving riboflavin- and pyridoxine-deficient rats of series II. The conclusion is drawn from the data that neither riboflavin nor pyridoxine deficiency has any appreciable influence on the acetylation of sulfanilamide under the conditions of the present experiment. This conclusion is based on the facts: (1) that there was no consistent decrease in acetylation as the deficiencies progressed; and (2) that there was little or no significant consistent increase in acetylation when the missing vitamin was administered. In pantothenic acid deficiency, on the other hand, decreases in acetylation of 50% or more are a common occurrence (Shils et al., '49), with an immediate response to adequate pantothenic acid therapy; 2 mg of calcium pantothenate injected intraperitoneally into pantothenic

TABLE 1

Acetylation of sulfanilamide by riboflavin-deficient and pyridoxine-deficient rats

		DAY OF EXPERIMENT [1]						
		37	64	99	113	120	129	137
Series I. Partial protocols								
Rat no.		*Controls* [2]						
76 C	Wgt. — gm	150	190	215	205	205	225	
	Acetylation %	31	37	35	34	37	30	
78 C	Wgt. — gm	90	140	175	160	160	170	
	Acetylation %	22	33	39	40	37	38	
82 C	Wgt. — gm	123	178	204	195	195	189	205
	Acetylation %	33	38	41	40	39	36	35
		Riboflavin deficiency period				*Repletion period* [3]		
84 R	Wgt. — gm	98	114	140	130	130 [4]	170 [5]	
	Acetylation %	18	26	24	36	26	32	
87 R	Wgt. — gm	88	116	130	126	126 [4]	133 [4]	160 [5]
	Acetylation %	21	29	32	31	32	30	35
90 R	Wgt. — gm	100	136	155	142	142 [4]	150 [4]	165 [5]
	Acetylation %	26	33	33	34	33	31	38
		Pyridoxine deficiency period				*Repletion period* [3]		
93 P	Wgt. — gm	114	137	144		145 [4]	175 [5]	
	Acetylation %	32	31	39		29	39	
98 P	Wgt. — gm	80	100	122	125	133 [4]	150 [4]	185 [5]
	Acetylation %	26	29	28	39	32	33	36
99 P	Wgt. — gm	96	120	135	134	120 [4]	140 [5]	
	Acetylation %	26	28	32	31	30	29	

Series II (Group mean ± standard error of the mean)

	Riboflavin deficiency period			*Repletion period* [6]	
No. of rats	58th day [1]	92nd day	114th day	121st day [4]	133d day [4]
	%	%	%	%	%
8	34.1±2.61	34.6±1.66	30.6±1.74	34.0±1.40	37.5±1.39
	Pyridoxine deficiency period			*Repletion period* [7]	
	86th day [1]	148th day	154th day	161st day [4]	168th day [5]
	%	%	%	%	%
6	34.7±2.42	31.9±1.92	30.2±2.71	34.5±1.71	38.3±2.16

[1] Counting from the day on which feeding of the deficient diets began.
[2] Food intake restricted beginning on 38th day.
[3] Missing vitamin restored to diet on 116th day.
[4] Diet intake restricted after vitamin restored to diet.
[5] Diet fed ad libitum for one week prior to acetylation test.
[6] Riboflavin restored to diet on 119th day.
[7] Pyridoxine restored to diet on 155th day.

acid-deficient rats three hours before beginning the acetylation test will cause a return to the control range.[6]

All of the deficient rats were restricted in their food allowance after being returned to the control diet; this was done in order to keep their weights comparable to those during the latter part of the deficiency period. After acetylation tests were made during this period of restriction, all of the animals except the riboflavin-deficient group of series II were allowed to eat ad libitum for one week, and then another acetylation test was made. No marked differences were noted between the restricted and ad libitum periods, but there was a definite tendency toward higher acetylation values as the animals increased their weight.

DISCUSSION

Our evidence to date indicates that pantothenic acid deficiency markedly reduces the acetylating ability of the rat, but that thiamine, riboflavin, and pyridoxine deficiencies have little or no effect. Riggs and Hegsted ('49) are in agreement that thiamine deficiency has little or no effect on acetylation; however, they found that in riboflavin deficiency acetylation of *p*-aminobenzoic acid (PABA) was significantly less than was the case with their controls when 5 mg or less were administered, but that pantothenic acid deficiency caused still less acetylation. When the dose of PABA was increased to 10 mg there was little or no difference between the percentage acetylated by controls, thiamine-, riboflavin- or pantothenic acid-deficient rats (approximately 40%), indicating that the overloading of the organism was obscuring differences in the ability of the tissues to acetylate. Our experience with sulfanilamide reveals that the tissues are capable of a higher rate of acetylation, since we have usually obtained 35 to 45% acetylation of a dose of 56 mg of sulfanilamide by normal young adult rats. On a molar basis, this is 4½ times as much aromatic amine as Riggs and Hegsted administered.

[6] Authors' unpublished data.

With 56 mg, and even with 80 mg, of sulfanilamide, a marked decrease in acetylation is found with pantothenic acid-deficient rats (Shils et al., '49); thus, at higher dosage levels, sulfanilamide would appear to be more sensitive than PABA for detecting a deficiency of pantothenic acid in the rat.

On the basis of data thus far obtained there is reason to believe that the extent of acetylation in non-pantothenic acid-deficient rats is related to weight. This point is being investigated further, but there is considerable evidence for this view. The amount of bromophenylmercapturic acid formation, for example, has been found to be related to weight (Stekol, '37). In well-nourished rats the weight of the liver is directly related to body weight by a power function (Dunn et al., '47). It would be expected that, where a conjugation reaction involves the liver (or any other organ or tissue whose size increases with body weight), the per cent conjugation of a given amount of substance would increase with size. It is of further interest to note that Harkness et al. ('49) recently reported that the amount of coenzyme A in the liver is directly proportional to the level of liver nitrogen.

Even though the acetylation-weight relation may not prove to be a sensitive one, nevertheless it should be considered in interpreting results. Where control animals are not restricted in weight to that of the deficient animals, and the weight differences are large, the acetylation of the former may be higher on the basis of weight alone. For example, litter-mate animals of series II on the control diet which were allowed to eat ad libitum acetylated 50 to 60% of the sulfanilamide when their weights reached about 300 gm, in contrast to the acetylation range of 25 to 40% of the lighter-weight deficient and repleted litter mates. Consequently, unless the control animals are restricted in weight to correspond to the deficient animals, it is necessary to have the latter act as their own controls by noting their response following restoration of the missing vitamin while restricting food intake, in order to maintain weight at the previous levels. The latter procedure was used in our experiments and no appreciable differences

were noted with the riboflavin- and pyridoxine-deficient rats before and after vitamin supplementation. Riggs and Hegsted ('49) did not mention whether their control group was restricted in food intake to restrain the normal weight gain, but they reported that a three-day fast had little influence on the acetylation of the controls. The lack of effect of a short-term fast is apparently different from the situation in which animals differ appreciably in weight, with corresponding differences in liver size and over-all conjugating ability.

SUMMARY AND CONCLUSIONS

1. Studies have been made of the ability of rats deprived separately of riboflavin and of pyridoxine to acetylate sulfanilamide. These deficiencies per se caused little or no significant decrease in acetylation, in contrast to the marked decrease found in pantothenic acid deficiency.

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EXPERIMENTAL LATHYRISM PRODUCED BY FEEDING SINGLETARY PEA (LATHYRUS PUSILLUS) SEED

JORDAN G. LEE

Department of Agricultural Chemistry and Biochemistry, Louisiana State University, Baton Rouge

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Lathyrism in humans and livestock has long been associated in India and the Mediterranean region with the prolonged ingestion of large quantities of seeds of the *Lathyrus* genus, especially *Lathyrus sativus.* That this genus is actually the cause of this condition has been denied by some, notably Anderson, Howard and Simonsen ('25). In recent years the emphasis on winter cover crops in the Gulf states of this country has led to the widespread planting in this region of the Singletary pea (*Lathyrus pusillus*) and the Caley pea (*Lathyrus hirsutus*). Cases of apparent lathyrism in livestock grazing fields containing these plants have been reported (Sugg, Simms and Baker, '44; personal communications to the author). These reports implicate the seed as the causative factor. This paper reports an investigation to determine whether the seeds of the Singletary pea are actually capable of producing the lathyritic syndrome and, if so, to obtain information as to the cause.

EXPERIMENTAL

Singletary peas having a guaranteed purity of 99% were obtained commercially and finely ground. Seeds of an edible soybean and of *Pisum sativum* were also obtained from com-

mercial sources and ground finely. These meals were incorporated at the 70% level into a test diet, whose composition is given in table 1.

The rations containing Singletary peas and *Pisum sativum* were fed to both albino rats and chicks. The feed containing the edible soybeans was fed only to albino rats. The chicks were week-old New Hampshires and were fed ad libitum in groups of 10 for a two-week period. The rats were of both sexes and varying weights (80 to 130 gm) and were fed ad libitum in groups of 4 for a 30-day period. Control groups of both species were fed the commercial feeds used in this laboratory as stock feeds.

TABLE 1

Composition of test diet

COMPONENT	AMOUNT BY WEIGHT	COMPONENT	AMOUNT BY WEIGHT
Singletary pea meal[1]	700	Bone meal	17
Wheat	100	Oyster shell, ground	10
Alfalfa leaf meal	90	Salt (1.7% $MnSO_4$)	10
Liver meal	50	Delsterol	3
Meat scraps	20		1000

[1] When fed, other seed meals replaced only the Singletary pea meal.

Only those animals receiving the Singletary pea diet showed any abnormalities at any time. On that ration the chicks almost immediately developed leg weakness and inability to walk or stand, lost weight and were all dead within the two-week period. Death was apparently due to inanition, and gross autopsy failed to reveal any changes incompatible with inanition. The rats fed the same ration developed within a 10-day period a shuffling gait, which gradually progressed to complete paralysis of the hindquarters over the 30-day period. Other noticeable symptoms in these animals were a curvature of the spine, exostoses of the long bones, incontinence and emaciation. No deaths occurred. On autopsy the exostoses were easily apparent; the bones were discolored and the

epiphyses of the long bones were greatly enlarged. The tibias especially developed a club-like appearance. Muscular atrophy was apparent. Since this original group was run, 9 completely paralyzed animals have been returned to stock

TABLE 2

Effects of various diets and dietary supplements in producing lathyrism

	DIET FED	DAYS FED	NO. RATS	RESULT
1.	Test + 10% casein [1]	30	2	Paralysis
2.	Test + 2% brewers' yeast [1]	30	3	Paralysis
3.	Test + 5% brewers' yeast [1]	30	4	Paralysis
4.	Test + 20 mg racemic alpha-tocopherol per day [2]	30	13	3 paralyzed totally 5 paralyzed partially 5 symptom free
5.	Test + 0.1 % gum guaiac [1]	30	3	Paralysis
6.	Test + 0.1% gum guaiac + 1% citric acid	25	3	Paralysis
7.	70% water extracted S. peas [3]	14	2	No symptoms
8.	70% ethanol extracted S. peas [3]	40	5	No symptoms
9.	70% S. peas steamed for 6 hours at 100°C.[3]	30	2	Paralysis
10.	Stock + ash from S. peas = 70% level of peas	30	2	No symptoms
11.	Stock + divicine sulfate at 1% level	30	2	No paralysis but depressed growth

[1] Added material substituted for an equal quantity of wheat in the test diet.
[2] Fed each day as individual supplement.
[3] Substituted in test diet for the raw Singletary pea meal.

feed and given a chance to recover. Only one of the group recovered the use of his legs.

To gain further information as to the cause of the paralysis, the diets indicated in table 2 were fed to albino rats of both sexes, ranging from 60 to 100 gm in size. The altered pea meals listed were substituted for the raw Singletary peas

in the test diet. The indicated additions were substituted for an equal quantity of wheat in the original test diet containing 70% raw Singletary pea meal. Results are indicated for each group.

RESULTS AND DISCUSSION

The syndrome described above is essentially the same as that described by others who have fed seeds of *Lathyrus odoratus* (Geiger, Steenbock and Parsons, '33; Lewis and Esterer, '43; Lewis et al., '48), *Lathyrus hirsutus* (Turney, Copeland and Salmon, '43; Lewis et al., '48), *Lathyrus sativus* (Stockman, '31) and *Lathyrus tingitanus* (Lewis et al., '48). It does not seem to tally at all with the syndrome described for *Lathyrus sylvestris* (Daniel et al., '46; Lewis et al., '48). The reports here cited, plus others not cited, make it abundantly clear that differences in specie of test animal, test conditions, specie and strain of peas, and growing conditions all greatly influence apparent toxicity. It is not surprising that varying claims can be found in the literature. A comparative discussion seems rather pointless until the toxicity of the various species has been more thoroughly and widely investigated.

In this case the purity of the seed used was such that it is difficult to believe that other seed present as contaminants could have produced such an effect. It is true that Anderson, Howard and Simonsen ('25) and Acton and Chopra ('27) have stated that lathyrism in India is caused not by *Lathyrus sativus* but rather by *Vicia sativa* seed, present as a contaminant. The data presented, however, indicate that it was necessary to feed vetch seeds at a 50% level to produce a paralysis. Vetch seeds are common contaminants of the *Lathyrus* seeds in this region, but not at such high levels; in the present experiments they were scarcely present.

The results obtained with the other seed meals and with the extracted Singletary pea meals indicate that the ration used is adequate and that some entity present in the peas is definitely toxic. The fact that the ash from Singletary peas

did not confer toxicity upon the stock diet indicates that an organic substance is the toxic material, although possible volatilization cannot be excluded. The failure of the heat treatment to destroy the factor indicates that an enzyme is not at fault. The possibility that an induced deficiency of protein or B-complex may be involved is not substantiated either by the syndrome characteristics or by the results obtained with casein and yeast feeding.

The deficiency of vitamin E in rats induced by feeding rancid fat (Fitzhugh, Nelson and Calvery, '44) resembles this paralysis to some extent. The fact that large supplements of synthetic alpha-tocopherol gave partial protection indicates the possibility that a relative deficiency of vitamin E may be involved in the development of the paralysis. It is difficult, however, to reconcile the pronounced skeletal changes and the large dosages required for even partial protection with the idea that a simple induced deficiency of vitamin E is at fault. The failure of the anti-oxidants to confer any protection seems to indicate that the effect of the tocopherol does not lie in its anti-oxidant effect in the intestine. It should be noted that Turney, Salmon and Copeland ('44) found that inclusion of as little as 10% *Lathyrus hirsutus* seed in the diet of rats caused reproductive failure, and that the feeding of alpha-tocopherol in unspecified amount did not negate this effect.

The causative factor present in any of the seed used experimentally to produce lathyrism has never been isolated and identified. The only claim for a specific compound is that of Anderson et al. ('25), who isolated divicine from their vetch seed and found it to be highly toxic when injected. They did not attempt to produce toxicity by feeding the pure compound. The parent nucleoside vicine seems not to be markedly toxic when injected (Flössner, '34; Herissey and Cheymol, '34).

In an investigation of the toxicity of pyrimidine derivatives, Kleiner ('12) tested divicine and certain related guanidine derivatives. He found the divicine to be highly toxic

when injected, and his description of the result tallies quite well with that reported by Anderson. He did not find any of these compounds to be toxic when fed in single doses. The divicine appears not to be absorbed readily, for he presents evidence that the urine did not contain either divicine or related structures and that the feces did contain some such material. No one has reported the isolation of either divicine or its parent nucleoside from any *lathyrus*, in so far as I am aware. The divicine sulfate fed in the present experiment was synthesized (Traube, 1893) and purified by the classical method. It was fed at an adequate level and for a sufficient length of time; if it is the cause of lathyrism some of the classical symptoms should have been found. The only untoward sign was a depressed growth rate. It is possible that the 1% level was too low or that the sulphate is much more insoluble, therefore less easily absorbed, than the naturally occurring form.

It should also be noted that divicine has been isolated in good yield (Schulze, 1891) from *Pisum sativum* as well as the vetches. With the exception of Stockman ('31), I know of no one who has induced toxic symptoms by feeding such seeds. Turney, Salmon and Copeland ('44) have reported that pigs developed leg weakness when grazed on Austrian winter peas (*Pisum arvense*) but these workers could not induce such symptoms in rats on diets containing 50% seeds. Geiger, Steenbock and Parsons ('33) and Lewis and Esterer ('43) failed to produce any untoward symptoms in rats fed diets containing 50% *Pisum sativum* seeds. A ration containing 70% *Pisum sativum* seeds failed to produce any symptoms in the present investigation.

While variation in strain and growing conditions might produce variation in divicine content, it seems as though some symptoms should have been noticed if divicine is a causative agent of such a paralysis in the rat. Further, Traube (1893) has reported divicine to be quite stable as the salt but unstable as the free base. Classically, it is prepared from vicine by acid hydrolysis. In experiments not here elaborated upon

I have found the causative agent in the Singletary pea to be quite unstable in an acid medium, much more so than in an alkaline one. Making all possible allowances, it seems that divicine cannot be the cause of the skeletal or muscular changes produced by the ingestion of Singletary peas.

SUMMARY

1. The ingestion of diets containing 70% Singletary pea (*Lathyrus pusillus*) seed meal leads to the development of the symptoms usually associated with lathyrism.

2. A deficiency within the ration is not the cause. The peas contain some toxic substance.

3. The toxicity is not due to the presence of an enzyme or toxic inorganic material.

4. The feeding of alpha-tocopherol in large doses confers some protection against the paralysis. This is not due to an anti-oxidant effect within the digestive tract.

5. Divicine is not the material present which is responsible for the skeletal and muscular changes.

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THE INFLUENCE OF DIET COMPOSITION ON VITAMIN B_{12} ACTIVITY IN MICE [1]

D. K. BOSSHARDT, WINIFRED J. PAUL AND R. H. BARNES
Department of Biochemistry, Medical Research Division, Sharp and Dohme, Inc., Glenolden, Pennsylvania

THREE FIGURES

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Vitamin B_{12} has been shown to be an essential nutrient for several animal species as well as for several microorganisms. Very little is known about the role of vitamin B_{12} in metabolic processes, although it has been shown to be a potent anti-pernicious anemia factor in humans (West, '48) and to be interrelated in some manner with the activity of choline in rats and chicks (Schaeffer, Salmon and Strength, '49a, b).

Animal responses to several of the B vitamins have been shown to be greatly affected by changes in dietary composition with regard to the three major foodstuffs; carbohydrate, fat and protein. The effects of variations in dietary composition on requirements for several of the B vitamins have provided information of prime importance in elucidating the metabolic function of these vitamins.

The present study was designed to investigate the effects of alterations in dietary composition on vitamin B_{12} activity in the growing mouse.

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EXPERIMENTAL METHOD

The diets employed consisted of 0.5% corn oil,[2] 0 to 52.5% hydrogenated cottonseed oil,[3] 20% casein,[4] 20% glucose,[5] 4% salt mixture (Jones and Foster, '42), and 2% cellulose.[6] Each diet was supplemented to contain, per 100 gm, 4 mg of α-tocopherol, 900 U.S.P. units of vitamin A, 180 U.S.P. units of vitamin D, 1 mg of 2-methyl-1,4 naphthoquinone diacetate, 0.8 mg of thiamine hydrochloride, 1.6 mg of riboflavin, 0.8 mg of pyridoxine hydrochloride, 4.0 mg of niacin, 4.4 mg of calcium pantothenate, 4.0 mg of para-aminobenzoic acid, 200 mg of choline chloride, 21 mg of inositol, 0.2 mg of folacin, and 0.02 mg of biotin. The remainder of each diet consisted of white dextrin.

Diets containing 0.5, 10.5, 20.5, 30.5, 40.5 and 53.0% fat were fed ad libitum to groups of 16 male albino weanling mice (Sharp and Dohme, Swiss Webster strain) for 15-day periods. One-half of the mice in each group were given 0.1 μg of crystalline vitamin B_{12} per day by intraperitoneal injection. The vitamin B_{12} was given in two equal doses on the first and 8th days of the test period. Records were kept of the body weight gains and of the food intakes.

In figure 1 are shown the average weight gains and the average food intakes expressed in calories. A reduction in the fat content of the diet to 0.5% resulted in a marked decrease in the growth rate. This retardation of growth was completely counteracted by the administration of vitamin B_{12}. In the absence of vitamin B_{12}, additions of fat to the diet resulted in increases in growth until the diets contained approximately 40% fat. No appreciable difference in growth was noted in the mice receiving vitamin B_{12} throughout this range of dietary fat levels. When diets containing 40% fat were fed, no vitamin B_{12} effect was noted. Fat levels in ex-

[2] Mazola.
[3] Primex.
[4] Vitamin-test, General Biochemicals, Inc.
[5] Cerelose.
[6] Cellu flour.

cess of 40% were detrimental, regardless of whether or not vitamin B_{12} was given.

A comparison of the food intake values, expressed as calories, suggested that the differences in growth observed could be attributed to differences in food intake, rather than to an increase in the economy of food utilization.

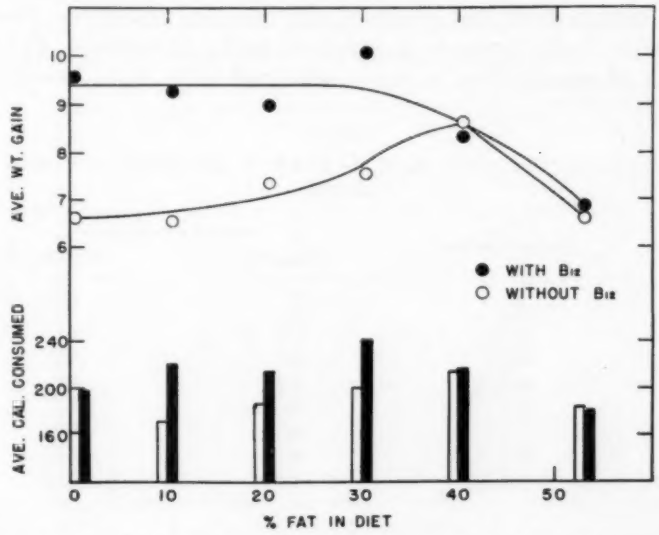

Fig. 1 The relationship between fat level in the diet and the 15-day weight gains and ad libitum calorie intakes of mice with and without vitamin B_{12}.

A controlled feeding study was run to check this point. The previous study was repeated, but instead of allowing the mice to eat ad libitum the food intake was controlled so that all mice consumed equicaloric quantities of food. The results, given in table 1, show no growth differences attributable to vitamin B_{12} or to the level of fat in the diet. It must be concluded, therefore, that the growth differences found in the previous study were due primarily to differences in food intake.

The effect of vitamin B_{12} on the growth rate of stock mice fed a low fat diet suggested the possibility of a simple mouse growth assay for vitamin B_{12}. Varying quantities of crystalline vitamin B_{12} were administered by intraperitoneal injection to male weanling mice fed ad libitum a diet containing 0.5% fat, for a 15-day period. The results obtained are shown in figure 2. A straight line log-dose response curve was obtained but the slope of the line was much too small to make it the basis of a practical assay procedure. For a basis of comparison, a curve obtained with male weanling

TABLE 1

The effect of fat and vitamin B_{12} on the growth of mice pair-fed with regard to calories

FAT LEVEL IN DIET	AVERAGE WEIGHT GAINS	
	Without B_{12}	With B_{12}
%	*gm*	*gm*
0.5	5.8	6.5
5.5	6.2	6.0
10.5	6.7	5.9
20.5	6.8	6.2
30.5	6.2	6.5
40.5	6.3	6.9
53.0	6.7	6.6
Average	6.39	6.37

mice fed a diet containing 27.0% fat and 0.5% of an iodinated casein with thyroid activity [7] (Bosshardt et al., '49) is shown. The difference in growth rate resulting from the administration of increasing amounts of vitamin B_{12} is very striking. Increasing the daily administration of vitamin B_{12} from 0.001 to 0.01 μg resulted in a growth increment during the 15-day test period of approximately 4 gm in the case of the thyroid-fed mice but of only 1 gm in the low fat group.

It has been shown (Bosshardt et al., '46) that the feeding of liver extracts counteracts the growth retardation of mice fed high-protein diets. It has also been found (unpub-

[7] Protamone, Cerophyl Laboratories.

lished results) that pure vitamin B_{12} exerts this effect. A study was made to investigate the influence of variations in both the fat and protein levels in the diet on the effect of vitamin B_{12} on the growth of mice.

Diets containing 10, 20, 30, and 45% protein and 0.5 and 27.0% fat were fed to groups of 16 mice for 15 days. Eight mice of each group were given 0.1 μg of crystalline vitamin B_{12} per day by intraperitoneal injection in two doses. The results of this experiment are shown in figure 3.

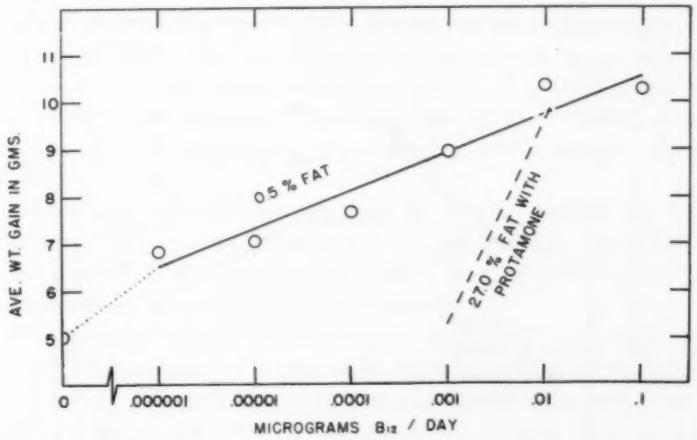

Fig. 2 A comparison of the 15-day growth response of mice receiving vitamin B_{12} by intraperitoneal injection when fed a low fat diet and when fed a 27% fat diet with Protamone.

With diets containing 10% protein there was no effect on growth resulting from the feeding of fat or from the administration of vitamin B_{12}. However, as the protein level in the diet was increased, with the fat level maintained at 0.5%, there was a gradual but steady decrease in the growth of the mice that did not receive vitamin B_{12}. The average weight change value for the group fed a diet containing 45% protein and 0.5% fat without vitamin B_{12} was — 1.3 gm; however, this weight change is the average of the weight losses of three

survivors of the group of 8. Deaths started to occur on the 6th day. The average weight loss of the mice at the time of death was 1.7 gm.

The addition of fat to the diets containing 20% or more protein resulted in a slight but definite increase in growth and completely eliminated fatalities. Growth was further improved by the administration of vitamin B_{12} to the mice fed the low fat diet. However, the addition of both fat and vitamin B_{12} resulted in growth rates comparable with the best that have been obtained in our laboratory.

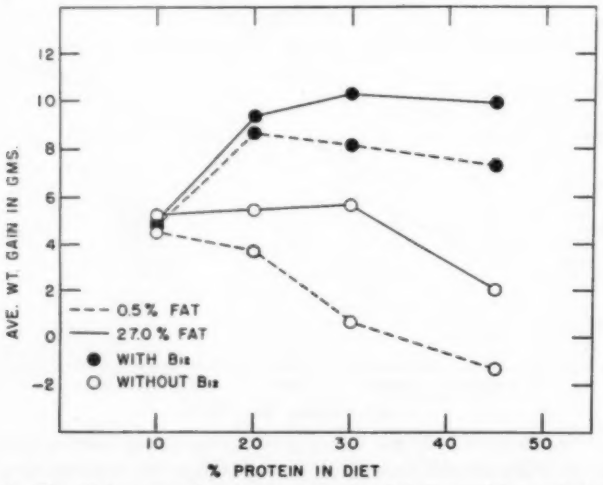

Fig. 3 The 15-day growth responses of mice fed diets with varying fat and protein levels with and without the administration of vitamin B_{12}.

DISCUSSION

Animal requirements for certain of the B vitamins have been shown to be influenced by the composition of the diet. It has been demonstrated that the feeding of fat-free diets not only intensifies, but is essential for the development of, the symptoms of a pyridoxine deficiency (Halliday and Evans, '37; Salmon, '38; Quackenbush and Steenbock, '38). The dermatitis produced by the feeding of a fat- and pyridoxine-free

diet may be improved by the feeding of either fat or pyridoxine, although both are essential for a complete cure.

Evans and Lepkovsky ('29) have shown that thiamine can be spared by increasing the fat level in the diet. This sparing effect may not be due to the increase in the level of fat in the diet, but rather to the corresponding decrease in carbohydrate. The metabolism of carbohydrate requires the presence of the thiamine-containing cocarboxylase in the tissues, whereas this coenzyme is not essential for the metabolism of fat.

MacKay and Barnes ('41) have shown that the inclusion of corn oil delays the development of acrodynia in the albino rat fed a diet containing dried egg white. The symptoms of the acrodynia were cured by either biotin and pyridoxine or by corn oil and pyridoxine, suggesting a biotin-sparing effect of fat.

Conversely, it has been reported by Evans, Lepkovsky and Murphy ('34), and by Mannering, Lipton and Elvehjem ('41) that increasing the level of fat in the diet of rats results in an increased requirement for riboflavin.

Interrelationships have also been shown to exist between dietary composition with regard to the major food components and dietary composition with respect to the other vitamins. The results of the present investigation extend these interrelationships to include protein, fat, carbohydrate and vitamin B_{12}.

For many years it has been recognized that fat, in excess of the quantity necessary to supply the essential fatty acids, has a beneficial effect on growth. Two publications in which a marked influence of fat was recorded (Deuel, Meserve, Straub, Hendrick and Scheer, '47; Lassen and Bacon, '49) were examined critically and it was noted that in neither case was an obvious source of vitamin B_{12} included in the diet. In view of the results reported here, it would appear that vitamin B_{12} if employed in place of the fat might have given the same results. If this concept is valid, it will be necessary to

reconsider the well established belief that fat, over and above the essential fatty acids, is necessary for optimum nutrition.

This interrelationship between fat and vitamin B_{12} may have far-reaching implications in the general field of human nutrition, especially in times of food shortages. Major nutrients in short supply in Europe during and after the two world wars were animal proteins, which are sources of vitamin B_{12}, and fat. It would appear that even with an adequate intake of calories and protein, primarily of plant origin, a shortage of fat in the diet would aggravate a potential vitamin B_{12} deficiency due to the lack of animal protein sources, or, at the same time, a shortage of vitamin B_{12} would intensify the deleterious effects of a low fat intake. A complete alleviation of these effects would, of course, necessitate adequate intakes of both fat and vitamin B_{12}.

Two possible metabolic functions of vitamin B_{12} are suggested. Vitamin B_{12} may, like thiamine, play an essential role in the catabolism of carbohydrate or of the carbohydrate-like residues of protein, or it may have a function in the conversion of intermediates formed in the metabolism of carbohydrate and protein to fats (other than the essential fatty acids) that are required for optimum growth. The fact that deleterious effects of a high protein diet may be overcome by vitamin B_{12} would suggest further a specific role of vitamin B_{12} in protein metabolism other than in the conversion of carbohydrate-like residues of fat.

Determination of the specific role of vitamin B_{12} in intermediary metabolism has become more complex because of the interrelation found to exist among vitamin B_{12}, choline and methionine (Schaeffer, Salmon and Strength, '49a, b) and also between vitamin B_{12} and pteroylglutamic acid (Heinle, Welch and Pritchard, '48).

SUMMARY

Variations in dietary composition with regard to fat, carbohydrate and protein have been shown to have an influence on vitamin B_{12} activity in the growing mouse.

A decrease in the fat level of the diet intensifies the growth retardation due to a deficiency of vitamin B_{12}. This growth retardation may be partially corrected by the feeding of fat or by the administration of vitamin B_{12}. These effects are intensified by increases in the protein level of the diet.

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FACTORS INFLUENCING THE UTILIZATION OF CAROTENE FOR STORAGE OF VITAMIN A IN THE RAT

M. G. VAVICH AND A. R. KEMMERER
Department of Nutrition, University of Arizona, Tucson

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Liver storage of vitamin A has been used to study the availability of carotene in various materials (Treichler, Kemmerer and Fraps, '42; Guggenheim, '44) and the effect of xanthophylls (Kemmerer, Fraps and De Mottier, '47; Kelley and Day, '48), of vitamin E (Johnson and Baumann, '48a), and of hypo- and hyper-thyroidism (Johnson and Baumann, '47b; Wiese, Mehl and Deuel, '48; Heimer, Maslow and Sobel, '49) upon carotene utilization, and for the bioassay of vitamin A (Foy and Morgareidge, '48). From this work it is apparent that factors within the animal itself affect vitamin A storage. Johnson and Baumann ('47b) attributed part of the increased retention of vitamin A found in the livers of rats fed thiourea and thiouracil to the small size of the animal. Heimer et al., ('49) conclude that the presence or absence of thyroid influences vitamin A storage to a large extent only to the degree that it affects the rate of growth.

Since liver storage of vitamin A is widely used for biological tests, it is imperative that factors within the animal that may affect these tests be given careful study. This paper reports on the effect of varying size and food consumption upon the utilization of beta-carotene for liver and kidney storage of vitamin A by the rat.

EXPERIMENTAL

The low vitamin A diet used in these experiments consisted of white corn meal 49.2, cottonseed meal 9.6, powdered skim milk 28.9, whole wheat 2.0, irradiated yeast 0.5, non-irradiated yeast 4.3, sodium chloride 0.5, calcium carbonate 0.5, ferric citrate 0.5, copper sulfate 0.5 and Wesson oil 3.5%. This diet contained less than 0.1 μg carotene per gram. After 35 to 40 days on this ration rats show typical symptoms of vitamin A deficiency. In order to determine whether this diet had any greater destructive effect on carotene than the U.S.P. vitamin A test diet, carotene in cottonseed oil [1] was incorporated into both diets to give a concentration of 60 μg per gram. They were stored for 15 days at refrigerator temperature, 8 to 10°C., room temperature, 25 to 27°C., and incubator temperature, 37°C. The retention of carotene was the same in both diets, 100% at 8 to 10°, 90% at 25 to 27° and 62% at 37°.

Animals of the Sprague-Dawley strain and of a strain of hooded rats [2] were used. When they were 12 to 14 days old the regular stock diet was replaced with the basal low vitamin A diet. At 28 days of age (40 to 50 gm) the litters were separated into pairs according to strain, sex and weight. This technique resulted in rats which had uniformly low vitamin A stores. The livers and kidneys of the rats, taken from the various litters, showed only 4 to 6 μg of apparent vitamin A per rat. For the determination of effect of size upon vitamin A storage, one of each pair was started immediately on 60 μg daily of beta-carotene equivalent in cottonseed oil.[3] The pair mate was fed 1.2 μg of carotene daily until it weighed approximately 100 gm; then it was placed on experiment and given 60 μg of beta-carotene equivalent daily. Analysis showed that 1.2 μg of carotene daily did not measurably affect the amount of vitamin A stored. The amount of basal diet was the same for each rat in the

[1] Wesson.

[2] Obtained from Dr. N. B. Guerrant, Pennsylvania State College.

[3] See footnote 1.

pair during the experimental period. For determining the effect of the amount of food consumed upon vitamin A storage one of each pair of rats received one-half as much food as its pair mate. Both received 60 μg of the beta-carotene equivalent daily in the cottonseed oil. All the rats were given the carotene supplement for 12 days and were killed on the 14th day.

Carotene solutions were prepared from a mixture of 90% beta- and 10% alpha-carotene by recrystallization from benzene with methyl alcohol. The amount of each isomer was determined by chromatographic adsorption on $Ca(OH)_2$. The alpha fraction was considered to have one-fourth the vitamin A activity of the beta fraction (Johnson and Baumann, '47a). The daily dose was fed directly to each rat from a dropper calibrated to deliver 60 μg of beta-carotene equivalent in 100 mg of cottonseed oil (1% alpha-tocopherol added). The rats were killed with chloroform and their livers and kidneys were removed, weighed, and immediately placed in 12% alcoholic KOH. After digesting for 30 minutes on a steam bath, the unsaponifiable fraction was extracted with peroxide-free diethyl ether. The ether extracts were dried and aliquots placed in Evelyn colorimeter tubes and evaporated to dryness under reduced pressure. The residue remaining was dissolved in 1 ml of dry chloroform and analyzed for vitamin A by the Carr-Price reaction. A drop of acetic anhydride was added to each tube prior to the addition of the $SbCl_3$ solution in order to prevent cloudiness.

DISCUSSION OF RESULTS

The data in table 1 show the effect of the size of the rat upon the amount of vitamin A stored in the liver and kidneys when carotene is fed. The smaller rats stored considerably more vitamin A in the liver than did their larger pair mates. Kidney storage of vitamin A, on the other hand, was higher in the larger rats. The combined liver and kidney storage of vitamin A was higher in the three groups of smaller rats than in the three groups of larger rats. The differences be-

tween the large and small rats are highly significant by analysis of variance (Snedecor, '46).

As is shown in table 2, the rats which received the greater amount of energy and grew at the much faster rate did not store significantly different amounts of vitamin A than did the rats which received only one-half as much energy and grew only one-twelfth as much. Thus the amount of basal diet consumed had no effect on the utilization of carotene. The data in table 2 also show that rate of growth had no effect

TABLE 1

Effect of size of rat upon storage of vitamin A in livers and kidneys

EXPERIMENT	NO. OF RATS	MEAN WT. OF RATS		VITAMIN A		
		Start of experiment	Gain in weight	Liver	Kidney	Total
		gm	gm	μg	μg	μg
1	6	48	41	76.4 ± 2.45[1]	1.8 ± .15	78.2 ± 2.37
	6	103	22	56.0 ± 3.56	6.5 ± .95	62.5 ± 5.14
2	10	50	27	61.4 ± 6.04	less than 0.5	61.4 ± 6.04
	10	99	7	44.2 ± 4.55	2.3 ± .83	46.5 ± 4.44
3	6	44	41	65.1 ± 4.15	2.1 ± .28	67.2 ± 4.24
	6	99	33	49.6 ± 4.45	4.3 ± 1.07	53.9 ± 3.85

[1] Standard deviation of mean.

on the amount of vitamin A stored. These results are of especial interest when compared with those of Johnson and Baumann ('48b), who found that rats restricted in growth by diets inadequate in calories retained more of their hepatic vitamin A than rats which grew normally. In the present experiments the rats that failed to grow presumably needed less vitamin A than those that were growing, and consequently were larger during most of the experimental period. The fact that the two groups stored equivalent amounts of vitamin A suggests a less efficient storage of vitamin A from carotene in the underfed group.

By the Carr-Price reaction, the livers from the control rats contained less than 10% as much vitamin A as those from the rats which received carotene. On the other hand, the kidneys of the control rats contained about 2 μg of vitamin A per rat, which is about the same amount as was found in the kidneys of the smaller rats which received carotene. The vitamin extract from the kidneys of control rats was examined spectrophotometrically. A typical vitamin A curve was not obtained. When the extract was chromatographed on calcium hydroxide and the chromatogram exposed to ultraviolet light, two fluorescent bands were noted. One of these bands was due to vitamin A. In our experiments the amount

TABLE 2

Effect of amount of food consumed upon storage of vitamin A in livers of rats

BASAL DIET FED	NO. OF RATS	WT. OF RATS		VITAMIN A STORED IN LIVER
		At start	Gain in wt.	
gm		*gm*	*gm*	*μg*
594	6	63	36	67.3 ± 4.56[1]
297	6	64	3	64.9 ± 4.45

[1] Standard deviation of mean.

of vitamin A in the kidneys was so small in comparison with the amount in the liver that it did not materially influence our findings.

The results of these experiments show one of the reasons why variable results are obtained in vitamin A storage experiments. The size of the experimental rats is one of the factors which should be carefully standardized.

SUMMARY

The size of the rats used as experimental animals markedly influenced the utilization of carotene for liver storage of vitamin A. When 60 μg of the beta-carotene equivalent were fed, the combined liver and kidney storage of vitamin A was higher in three groups of rats averaging 44 to 50 gm than it

was in three groups of rats averaging 99 to 103 gm. The amount of basal diet consumed had no effect on the vitamin storage.

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STUDIES ON THE ENLARGED HOCK DISORDER (PEROSIS) IN TURKEYS

M. L. SCOTT

Agricultural Experiment Station and the School of Nutrition, Cornell University, Ithaca, New York

THREE FIGURES

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One of the difficulties often encountered in raising turkeys, especially under confined conditions, is the development of abnormal hocks in a number of the largest male turkeys. This condition, which may or may not be identical with the perosis of chicks, is characterized by an enlargement and swelling of the hock joint, and a bending and often twisting of both the tibia and metatarsus, resulting usually in a bowing of the legs.

The fact that in many cases the tendon of Achilles does not slip from its condyle, together with the fact that the condition is most often observed in the largest male turkeys after they are 8 weeks of age, indicates a lack of identity with typical perosis. However, since the condition is similar in many respects to perosis, it will be referred to as such in this report.

During the course of studies on the "animal protein factor" it was noted that poults receiving diets deficient in animal protein developed slightly enlarged hock joints at about two weeks of age. While this condition was in no way disabling and, indeed, tended to disappear as the poults approached 4 weeks of age, it occurred to us that perhaps the hock joints in these poults had been weakened during this early growth period to the extent that they could not adequately support the weight of the birds as they grew heavier. Continued observation of these birds, which recived a normal growing

ration after 4 weeks of age, showed that this was apparently the case, since many of the turkeys which had shown slightly enlarged hocks at two weeks of age developed severe hock enlargements and bowed legs at 8 to 14 weeks of age.

Manganese, choline, biotin, niacin, folic acid, riboflavin and pantothenic acid have all been shown to have some effect upon perosis. However, the diets used in these studies contained levels of all of these factors which were equal to or greater than the levels generally recognized to be required by turkeys. On the other hand, results obtained by Gillis and Norris ('49a, b), using chicks, show that the requirement for methylating agents such as choline and betaine is related to the vitamin B_{12} content of the diet. In earlier studies with poults Scott, Heuser and Norris ('48) and Scott ('48) found that the amino acid, glycine, was effective in replacing a part of the growth-promoting activity of fish meal and meat scrap.

Experiments were designed, therefore, to determine whether or not glycine, choline, betaine, and vitamin B_{12}, in any of various combinations, were effective in the prevention of perosis in turkeys.

EXPERIMENTAL

Broad-breasted bronze poults of mixed sexes obtained from a commercial hatchery were used in all experiments, 15 to 18 poults per lot. The poults were housed in thermostatically controlled heated pens having raised wire floors.

Experiment 1

Using the basal diet presented in table 1, an experiment was conducted in which choline, betaine and glycine were fed in various combinations with and without the addition of vitamin B_{12}. The experimental plan and results are shown in table 2. It is apparent from these results that the addition of either choline or betaine, alone or with vitamin B_{12}, improved perosis to a considerable degree. On the other hand, the addition of choline, glycine and vitamin B_{12} together or choline, betaine, and vitamin B_{12} together completely pre-

TABLE 1

Animal protein-deficient basal ration

INGREDIENT	AMOUNT
	%
Soybean meal	55
Corn meal	15
Ground wheat	13
Rolled oats	10
Dicalcium phosphate	3.7
Calcium carbonate	1.5
Salt, iodized	0.8
Manganese sulfate	0.025
Fish liver oil	1.0
Riboflavin	2 mg per lb.
Vitamin K (menadione)	2 mg per lb.
Niacin	12.5 mg per lb.
Protein	28
Methionine	0.61
Cystine	0.42
Arginine	1.54
Glycine	1.75

TABLE 2

Effect of choline, betaine, glycine and vitamin B_{12} upon growth and perosis

(Experiment 1)

TREATMENT	GROWTH (4 WKS.)		PEROSIS (4 WKS.)	
	Without B_{12}	With B_{12} [1]	Without B_{12}	With B_{12} [1]
	gm	*gm*	%	%
None [2]	427	426	39	30
Choline, 0.2%	445	450	17	25
Betaine, 0.2%	440	475	12	6
Glycine, 0.5%	467	476	33	18
Choline + betaine	435	460	30	0
Choline + glycine	444	455	0	0
Betaine + glycine	459	464	17	0

[1] Vitamin B_{12} added at a level of 2 μg/100 gm as concentrate (Pfizer Bi-Con — A.P.F.–6).

[2] Basal diet contained approximately 850 mg choline per pound and 1.75% glycine.

vented perosis. None of the treatments had any significant effect upon growth.

Experiment 2

The basal diet used in the first experiment was modified for the second experiment in order to make it more deficient in choline and glycine. This was done by substituting 12% of vitamin-free casein [1] and 8% of corn meal for 20% of the

TABLE 3

Effect of choline, betaine, glycine and vitamin B_{12} upon growth and perosis

(Experiment 2)

TREATMENT	GROWTH (4 WKS.)		PEROSIS (4 WKS.)	
	Without B_{12}	With B_{12} [1]	Without B_{12}	With B_{12} [1]
	gm	*gm*	%	%
None [2]	476	450	67	83
Choline, 0.2%	469	421	61	33
Betaine, 0.2%	445	442	61	50
Glycine, 0.5%	455	440	50	50
Choline + betaine	404	448	33	16
Choline + glycine	449	473	50	11
Betaine + glycine	445	439	41	61
Fish meal, 5%, + meat scrap, 5%	453	...	22	..

[1] Two micrograms vitamin B_{12} per 100 gm diet as concentrate.

[2] Basal diet contained approximately 650 mg choline per pound and 1.2% glycine.

soybean meal. The modified diet contained approximately 650 mg of choline per pound and 1.2% glycine.

The results of this experiment, presented in table 3, show the same general trend as those obtained in the first experiment. However, in this experiment the combination of glycine and choline did not show any marked effect upon perosis unless vitamin B_{12} was also added. It is of interest that the addition of vitamin B_{12} alone to the basal diet increased the incidence of perosis. None of the additions to this diet, including that of 5% of fish meal and 5% of meat scrap, com-

[1] General Biochemicals, Inc.

pletely prevented perosis. As in the previous experiment, growth was not affected materially by any of the treatments.

Experiment 3

For the third experiment, the basal diet was made even more deficient in choline and glycine by the substitution of 18% of casein for soybean meal, corn meal being used to maintain the protein at a comparable level. Two micrograms of vitamin B_{12} were added per 100 gm of basal diet. This

TABLE 4

Effect of choline and glycine upon growth and perosis

(Experiment 3)

TREATMENT	GROWTH (4 WKS.)	PEROSIS (4 WKS.)
	gm	%
None [1]	466	100
None	473	82
Choline, 0.1%	447	100
Choline, 0.1%	417	60
Glycine, 0.5%	474	92
Glycine, 0.5%	449	86
Choline + glycine	469	46
Choline + glycine	426	60

[1] Basal diet contained approximately 420 mg choline per pound, 0.85% glycine, and 2 μg vitamin B_{12} per 100 gm added as concentrate.

diet contained 420 mg of choline per pound and 0.85% glycine. The results of this experiment, presented in table 4, show that the greatest improvement in perosis was again obtained when choline and glycine were added together. However, the highest levels used in this experiment were 820 mg of choline per pound of diet and 1.35% glycine, which obviously were not sufficiently high to have any marked effect upon the condition.

Experiment 4

For this experiment the same basal diet was used as was employed in experiment 3, except that in this case the diet

was also supplemented with calcium pantothenate, 2 mg per pound, and biotin, 25 μg per pound. Three choline levels were used: (1) 420 mg per pound (approximately one-half the National Research Council allowance for turkeys); (2) 920 mg per pound (the N. R. C. allowance which is in agreement with the studies of Evans et al., '42 and Evans, '43); and (3) 1,920 mg per pound (more than twice the N. R. C. allowance). At each level of choline, three levels of glycine were used: 0.85%, 1.85% and 2.85%. Sarcosine, betaine, and creatine

TABLE 5

Effect of various supplements upon prevention of perosis at different levels of choline

(Experiment 4)

SUPPLEMENT	GROWTH (4 WKS.)			PEROSIS (4 WKS.)		
	0.1% Choline	0.2% Choline	0.4% Choline	0.1% Choline	0.2% Choline	0.4% Choline
	gm	*gm*	*gm*	%	%	%
None [1]	489	503	539	83	64	40
Glycine, 1%	503	551	487	100	50	25
Glycine, 2%	497	468	488	100	8	0
Betaine, 0.2%	475	544	...	100	38	..
Sarcosine, 0.4%	...	547	...	...	15	..
Creatine, 0.4%	...	505	...	...	15	..
Fish meal, 5%, + meat scrap, 5%	...	476	...	...	0	..

[1] Basal diet similar to that used in experiment 3.

were also added to the diet containing 920 mg of choline per pound. One lot of birds also received this level of choline plus 5% fish meal and 5% meat scrap. The results of this experiment are presented in table 5.

These results clearly demonstrate an interrelationship between choline and glycine in the prevention of perosis in poults. Betaine, at the level used, was completely ineffective in reducing the incidence of perosis when added to a diet deficient in choline. Betaine, however, caused a pronounced reduction in perosis when added together with a normal level of choline. Both sarcosine and creatine appeared to be more

active than glycine in reducing perosis when fed together with a normal level of choline. Five per cent of fish meal plus 5% of meat scrap completely prevented perosis when added to the diet containing 920 mg of choline per pound. These levels of fish meal and meat scrap supplied only 0.45% glycine, according to microbiological analysis by the method of Henderson and Snell ('48). The addition of either glycine or betaine to the diet containing a low level of choline increased perosis.

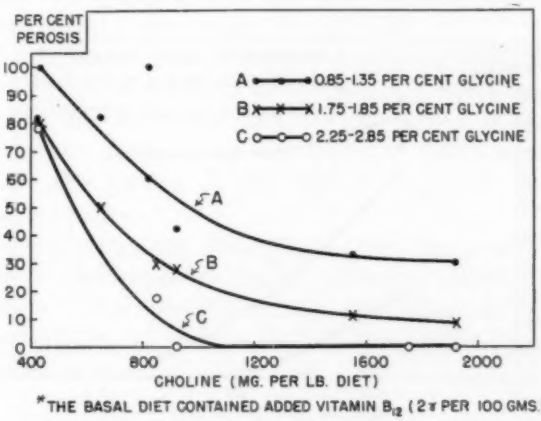

Fig. 1 Effect of choline upon perosis in turkey poults at different levels of glycine.

Since additions of choline produced no consistent increases in growth it appears that much less choline is required for growth in turkeys than is required for the prevention of perosis under these conditions.

Summary of all results

A composite summary of the results of all 4 experiments comparing levels of choline and glycine is presented in figures 1 and 2. These results show that perosis in turkeys could not be prevented by choline under the conditions of these

experiments unless unusually high levels of glycine were also present in the diet. For example, when the glycine content of the diet was 0.85 to 1.35%, which includes the level reported by Kratzer and Williams ('48) to be required by poults, the addition of choline up to twice the normal level produced no appreciable improvement in perosis over that obtained at a normal level (curve A, fig. 1). On the other hand, perosis was prevented by an almost normal level of choline when the glycine content of the diet was above 2.25% (curve C, fig. 1 and curve B, fig. 2).

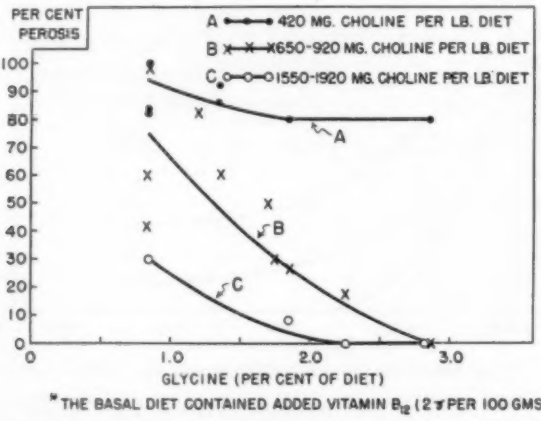

Fig. 2 Effect of glycine upon perosis in turkey poults at different levels of choline.

DISCUSSION

The results presented in this report show, as have the findings of others, that choline is a factor of primary importance in the prevention of perosis in turkeys. In no instance was it possible to prevent perosis completely as long as the choline level was below 850 mg per pound of diet. The importance of choline for the prevention of perosis in turkeys was first shown by Jukes ('40a). On the other hand, choline alone, up to levels over twice that recognized as the requirement, failed to prevent perosis when added to a diet

containing vitamin B_{12} but containing no fish meal or meat scrap. The addition of fish meal and meat scrap to a diet containing a normal level of choline completely prevented perosis, whereas these supplements did not prevent perosis completely when the diet contained only 650 mg of choline per pound.

In the absence of fish meal and meat scrap, perosis could be prevented by a combination of choline, glycine, and vitamin B_{12}. However, the levels of choline and glycine required for this effect were far in excess of those required when fish meal and meat scrap were present in the diet.

Thus it appears that fish meal and meat scrap contain a required factor in addition to vitamin B_{12} which can be synthesized in the turkey through the interaction of choline and glycine in the presence of vitamin B_{12}. Tests conducted to determine the nature of this factor have not yet yielded conclusive results.

The evidence in one direction favors the possibility that creatine is the required factor. However, strong evidence also exists of the possibility that the factor is betaine. A third possibility, of course, is that the factor is neither of these substances.

Evidence for creatine as the required factor

The results presented in this report show that creatine was effective in markedly reducing perosis when supplied together with a normal level of choline and added vitamin B_{12}.

The effectiveness of glycine, therefore, may possibly be explained by its conversion to creatine, a mechanism which is well known to occur in chicks (Hegsted et al., '41; Almquist et al., '41). Almquist et al. ('40, '41) have shown that creatine and its precursors possess growth-promoting effects for chicks when supplied together with adequate choline and methionine. They found creatine to be more effective than glycine on a molar basis, indicating that glycine is not converted quantitatively to creatine. Jukes ('41a) found creatine to be without

effect in the prevention of perosis in turkeys. However, in his work the creatine was added to a diet deficient in choline.

Evidence for betaine as the anti-perotic factor

Betaine was shown, in several experiments, to produce a pronounced anti-perotic effect when supplied together with a normal level of choline and with added vitamin B_{12}. The recent researches of Dubnoff ('49a, b) indicate that the choline oxidase necessary for conversion of choline to betaine is lacking in the livers of the guinea pig, rabbit and chick. If this is true also for the turkey, it would be logical to assume that choline cannot serve directly for certain body metabolic functions requiring betaine, if any such should exist. The results of Almquist and Grau ('44) show that choline has at least two effects in the chick which enhance growth, one of which can be replaced by arsenocholine while the other is replaced by betaine. Jukes ('40b) showed that arsenocholine almost prevented perosis in turkeys in place of choline under his experimental conditions. It is possible that in the case of the turkey the requirements are somewhat higher, and that therefore excessively high levels of choline are required in the absence of betaine or its immediate precursors.

Possible mechanisms for the interconversion of glycine, sarcosine, creatine and betaine

If one assumes that sarcosine can be methylated easily to produce betaine, one can plot the perosis-preventing effect of these materials in terms of betaine equivalents, as is shown in figure 3. The almost straight line obtained indicates that these two compounds are equivalent in activity. According to the curve presented in figure 3, the requirement of either betaine or sarcosine for complete prevention of perosis would be approximately 0.6%. The fact that creatine is as effective as sarcosine indicates, perhaps, that the poult has no difficulty in transforming creatine into sarcosine, a reaction which has long been known to occur.

The question arises as to the mechanism of action of glycine in preventing perosis. Assuming, again, that glycine must be converted to betaine, the results of the present experiments indicate that this conversion does not occur by direct quantitative methylation, since considerably more glycine than sarcosine, creatine, or betaine was required for the same degree of perosis-prevention.

Jukes ('41b) has shown that the chick, unlike the rat, is incapable of methylating aminoethanol. On the other hand,

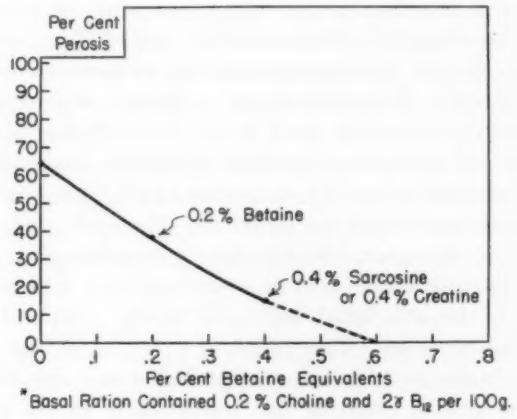

Fig. 3 Effect of betaine, sarcosine, and creatine upon perosis.

Jukes, Oleson and Dornbush ('45) showed that either monomethylaminoethanol or dimethylaminoethanol could be methylated and converted to choline by the chick. If an analogous situation exists for aminoacetic acid (glycine) and monomethylaminoacetic acid (sarcosine) in the formation of betaine, this would explain why sarcosine is as active as betaine in the prevention of perosis in turkeys, while glycine is not.

If the analogy holds, and glycine cannot be methylated directly, a possible explanation of its activity is that it is first converted to creatine which, in turn, is changed to sarcosine, which can then be methylated directly to form betaine.

duVigneaud and associates ('39) and Borsook and Dubnoff ('40b) have shown that the methyl groups used in the synthesis of creatine must come from methionine. However, the latter authors ('45) also showed that choline can supply these methyl groups indirectly by the intermediate formation of methionine from homocystine. This may help to account for the fact that in the studies presented here an exceedingly high level of choline was required in addition to glycine for the prevention of perosis.

If one assumes that creatine is the required factor, one must offer a mechanism for the conversion of betaine and sarcosine to creatine. Stetten ('41) and duVigneaud et al. ('46) have shown that betaine can be reduced to glycine in the animal body. However, since in the present experiments betaine was considerably more active than glycine in preventing perosis but no more active than sarcosine, one must assume that this change stops at sarcosine, and that sarcosine is then readily converted to creatine. Borsook and Dubnoff ('40a) have, however, shown that sarcosine plus arginine produces guanidoacetic acid and not creatine, indicating that sarcosine is demethylated before it reacts with the amidine group from arginine. Therefore, if creatine is the required factor, the activity of betaine is probably not due to its conversion *in toto* to creatine but more likely to its ability to furnish readily available methyl groups for the synthesis of creatine from glycine and arginine. The recent discovery by Muntz ('50) that the product derived from choline in the methylation of homocysteine to form methionine is dimethylglycine rather than dimethylaminoethanol, indicates that choline must be converted to betaine before methyl transfer can occur. In view of the findings presented by Muntz, the studies conducted by Gillis and Norris ('49a, b), together with those presented here, indicate the possibility that vitamin B_{12} is concerned in the oxidation of choline to betaine.

Jukes ('41c) and others have presented results showing that creatine and its precursor, glycine, produce perosis in chicks when added to diets deficient in choline. In the results

presented in this report both glycine and betaine intensified perosis when added in the absence of sufficient choline. It is indeed interesting that these substances are required to prevent perosis in turkeys when the diet contains adequate choline.

SUMMARY

1. The results presented in this report further emphasize the primary importance of choline in the prevention of perosis in turkeys. In no instance was perosis completely prevented as long as the choline level in the diet was below 850 mg per pound.

2. When animal protein was omitted from the diet, choline was ineffective in completely preventing perosis unless the diet also contained betaine or a relatively high level of glycine and vitamin B_{12}.

3. Both sarcosine and creatine were as active in the prevention of perosis as betaine, and more active than glycine, under similar conditions. The possible interconversion of these substances during metabolism is discussed.

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THE UTILIZATION BY THE ADULT RAT OF AMINO ACID MIXTURES LOW IN LEUCINE[1]

JOSEPH T. ANDERSON AND E. S. NASSET

Department of Physiology and Vital Economics, University of Rochester School of Medicine and Dentistry, Rochester, New York

ONE FIGURE

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The biological value or nitrogen balance index of a protein is evidently fixed by its content of one limiting essential amino acid. Mitchell and Block ('46) reported such a relationship for proteins low in isoleucine, lysine, methionine, and cystine plus methionine. Anderson and Nasset ('48) found the same condition to prevail with regard to amino acid mixtures low in isoleucine and methionine.

The present investigation demonstrates that a low level of leucine impairs the quality of an amino acid mixture for maintenance of N equilibrium in the adult rat and that D-leucine is utilized to some extent for this purpose in the presence of a small but inadequate supply of L-leucine.

METHODS

One group of adult male albino rats (series 120) served as subjects in all of the experiments described in this paper. At the beginning they were 120 to 130 days old and after a 48-hour fast weighed 180 to 212 gm. The animals were numbered in order of decreasing body weight. The sequence of feedings for the odd-numbered rats was as follows: (1) 12.5

[1] This investigation was supported in part by a grant from the Office of Naval Research. Acknowledgment is made to Merck and Company, Hoffmann-LaRoche, Inc., and the Lederle Laboratories for gifts of amino acids and vitamins, amino acids, and folic acid, respectively.

days of recovery diet (10% egg protein); (2) 7 days of nitrogen-free (N-free) diet; (3) 7 days of amino acid "full nitrogen" (full-N) diet; (4) 7 days of amino acid "half nitrogen" (half-N) diet; (5) 1.5 days of fasting. The sequence for the even-numbered rats was the same except that the order of "full" and "half" nitrogen periods was reversed. This cycle of feedings was repeated 5 times, using the following amino acid mixtures in the order given: I, "Complete;" II, one-third L-leucine; III, one -sixth L-leucine; IV, one-third DL-leucine; V, "Complete."

The recovery, N-free, and "complete" amino acid full-N diets were described in detail in a previous paper (Anderson and Nasset, '48, tables 1 and 2). The "complete" amino acid mixture simulates whole egg protein in that it contains the same amount of each naturally occurring essential amino acid per gram of total nitrogen. The non-essential amino acids of egg protein are replaced by the D-forms of 6 essential amino acids, plus sufficient L-glutamic acid to make the same total nitrogen. The full-N diets contain approximately enough of the amino acid mixture to maintain nitrogen equilibrium. The half-N diets were prepared by omitting half the quantity of each amino acid and adding an equal weight of sucrose. Each rat was fed the same estimated daily allowance of calories. The computation, Calories $= 121\ kg^{\frac{3}{4}}$, was based on the initial average fasting body weight of 0.196 kg. The one-third L-leucine amino acid mixture differed from the "complete" mixture by the omission of two-thirds of the L-leucine and its replacement by an isonitrogenous amount of L-glutamic acid. The slight change in caloric content was corrected by adjusting the amount of sucrose in the diet. The one-sixth L-leucine diet was prepared in analogous fashion. The one-third DL-leucine amino acid mixture differed from the one-third L-leucine mixture by the substitution of an equal amount of the racemic form for the natural isomer.

The dry diet was mixed with water equalling about 43% of its weight and fed by stomach tube in all but the recovery periods. The morning feeding was started at 9:00 A.M. On

evenings preceding an energy metabolism test or the beginning or ending of a urine collection period the second daily feeding was started at 9:00 P.M., but on other days at 4:00 P.M.

The syrupy diet mixture was dispensed from a brass pump 12 inches long and with a ¾-inch inside diameter containing a piston sealed by a leather washer. The piston was driven by a nut which traveled on a steel screw 16 inches long and 5/16 inches in diameter, with 24 threads per inch. The contents of the pump were stirred before delivery of each portion by pulling a ½-inch steel ball to and fro within the tube by means of an Alnico magnet placed on the outside.

Nitrogen intake was determined from analysis of the dry diet and the weights of portions of the syrupy diet delivered from the brass pump used in feeding the rats. The analyses of excreta and other methods employed were described in a previous paper (Anderson and Nasset, '48).

RESULTS

The nitrogen intake, fecal nitrogen, and urinary nitrogen in the various dietary periods are presented in table 1. These data are averages from equal numbers of odd rats and even rats and are therefore not influenced by any difference due to the order of feeding the half-N and full-N diets. It is interesting that feeding a diet containing amino acids, whether full or half N, after a N-free period often results in a diminished output of nitrogen in the feces. The probability values listed at the bottom of table 1 show that the output of fecal nitrogen was significantly decreased in 5 experiments. The increases shown for experiment I are not significant. Whenever fecal nitrogen excretion is diminished by the feeding of an amino acid mixture or a protein, the computed true digestibility or absorption of nitrogen will exceed 100%. The assumption that "endogenous" or "metabolic" nitrogen remains constant in this type of experiment is, therefore, obviously unjustified. When an amino acid mixture is fed as a source of nitrogen, digestion is not a prerequisite for absorption and it is probably more logical to deal with nitrogen

intake rather than true digestibility or absorbed nitrogen computed in the conventional manner.

The index of the quality of an amino acid mixture, used previously (Anderson and Nasset, '48), is the nitrogen balance index of absorbed nitrogen (the K of Allison and Anderson, '45), which is the rate of change of nitrogen balance with

TABLE 1

Average nitrogen balance data for rats receiving amino acid mixture diets

Experiment number	I	II	III	IV	V
Amino acid mixture	Complete	⅓ L-leucine	⅙ L-leucine	⅓ DL-leucine	Complete
Number of rats	12	10	12	10	12
Body weight (kg)	0.199	0.195	0.197	0.193	0.197
Metabolic body size ($kg^{¾}$)	0.297	0.294	0.295	0.291	0.296
	N BALANCE DATA (MG N/DAY/$KG^{¾}$)				
N-free period					
N intake	2	2	2	2	2
Fecal N	34	39	37	34	33
Urinary N	156	124	122	143	129
Half-N period					
N intake	75	76	76	78	76
Fecal N	38	38	33	34	29
Urinary N	108	101	97	95	90
Full-N period					
N intake	145	146	145	148	146
Fecal N	39	30	26	28	28
Urinary N	112	114	129	114	94
P [1] for fecal N difference					
N-free vs. half-N periods	0.10 [2]	0.15	0.03	1.0	0.0001
Half-N vs. full-N periods	0.9 [2]	0.003	0.003	0.0003	0.6

[1] Probability that the difference indicated was due to chance.

[2] In these instances increase in N intake caused increase in fecal N.

respect to absorbed nitrogen. Since in the present experiments the absorbed nitrogen values often exceeded the nitrogen intake, it seemed advisable to avoid their use. Hence in place of K in these experiments the nitrogen balance index of dietary nitrogen (the K' of Allison) is used. K' is the rate of change of nitrogen balance with respect to nitrogen intake.

K' is the product of $K \times$ digestibility, and if the amino acids of the diet are actually completely absorbed, K' and K are equal.

The nitrogen balance was computed for each dietary period and is plotted as a function of N intake in figure 1. The points representing the three dietary periods of each experiment are connected by lines. It will be noticed that nitrogen balance is not a linear function of nitrogen intake in any case.

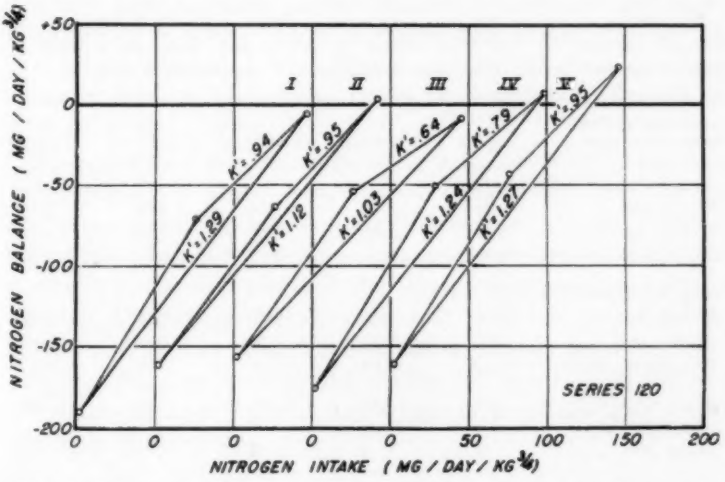

Fig. 1 Nitrogen balance index of ingested nitrogen for low-leucine amino acid mixtures.

Expt. I — "complete" amino acid mixture.
Expt. II — one-third L-leucine amino acid mixture.
Expt. III — one-sixth L-leucine amino acid mixture.
Expt. IV — one-third DL-leucine amino acid mixture.
Expt. V — "complete" amino acid mixture.

The increase in nitrogen balance per unit increase in nitrogen intake (K') is the slope of the line joining any two points.

In order to find whether the order of feeding the half-N and full-N diets made any difference in the results, K' values were computed from the odd rats and from the even rats separately. The results are given in table 2. In 10 compari-

sons only three are different (P = 0.05, 0.03, 0.015). It is concluded tentatively, therefore, that after one week of a N-free diet the excretion of urinary nitrogen on diets containing either half or the full maintenance requirement of nitrogen is consistent and reproducible for two weeks, and that no further account of the order of feeding need be taken in interpreting these experiments.

TABLE 2

The relation of order of feeding diets to values obtained for the N balance index. Odd rats received the full-N diet before the half-N diet; even rats received the diets in opposite order. Computed maintenance N requirements (NI_e).

Experiment number Amino acid mixture	I Complete	II ½ L-leucine	III ⅛ L-leucine	IV ½ DL-leucine	V Complete
Using N-free and full-N periods					
K' odd rats	1.22	1.14	1.04	1.25	1.28
K' even rats	1.36	1.07	1.02	1.24	1.27
P [1] (odd vs. even)	0.03	0.3	0.7	0.9	0.9
Using half-N and full-N periods					
K' odd rats	0.84	1.03	0.64	0.72	0.95
K' even rats	1.03	0.87	0.64	0.86	0.96
P [1] (odd vs. even)	0.015	0.08	1.0	0.05	0.9
NI_e [2]	152	143	161	140	122
Stand. dev. of mean NI_e	3	3	3	2	2

[1] P = probability that the indicated difference arose from chance alone as computed by the Student "t" test.

[2] NI_e = computed nitrogen intake required for nitrogen equilibrium.

Results using N-free diet period as the reference point

Consider first the K' values of figure 1 obtained by comparison of a N-free diet and a full-N diet (the lowest and highest points of a figure on the graph). The values 1.29 and 1.27, obtained using the "complete" amino acid mixture, agree well with each other, although 25 weeks intervened between experiment I and experiment V. These values were obtained by the same type of comparison used in the previous paper and the present results fall within the range previously

found for the same "complete" amino acid mixture. Using K' from the N-free and full-N diets as an index of quality, the amino acid mixtures tested fall in the order listed in the left half of table 3. The one-sixth L-leucine amino acid mixture was significantly poorer than the "complete" or the one-third DL-leucine mixture and perhaps poorer than the one-third L-leucine mixture ($P = 0.09$). The one-third L-leucine amino acid mixture was significantly poorer than the "complete" mixture and perhaps poorer than the one-third DL-leucine mixture ($P = 0.06$). As was found previously, the K' (or K) values computed from N-free and full-N diets came out greater than unity, which seems to indicate utilization of N greater than 100%.

It seems that K' values greater than unity arise from an extraordinarily great excretion of urinary nitrogen by the rat when receiving a N-free diet as compared with nitrogen excretion when the diet contains a small amount of a protein, a mixture of amino acids, or the single amino acid methionine (Brush, Willman and Swanson, '47). High urinary nitrogen in the N-free diet period causes an unusually large negative nitrogen balance, thus giving a very low position to the zero N-intake points in figure 1 and a high slope to the lines drawn through these points.

Results avoiding use of N-free diet period

If the excretion of urinary nitrogen by rats receiving a N-free diet is subject to a stimulatory influence not present when protein or an amino acid mixture is ingested, it would seem desirable to plan a method of protein or amino acid assay which does not utilize the urinary nitrogen value of the N-free period. In this study such a method is provided by comparison of the half-N and full-N dietary periods for each amino acid mixture. This comparison is expressed as the K' value or nitrogen balance index of dietary nitrogen, and is given by the slope of the line joining the two highest points from each experiment in figure 1.

The nitrogen balance index obtained by this method is 0.94 and 0.95 for two trials with the "complete" amino acid mixture. These values indicate that the amino acid nitrogen added to the half-N diet in making the full-N diet was approximately 95% utilized toward the attainment of N equilibrium. There is no theoretical objection to a utilization value of 0.95. The K' values yielded by this method for the amino acid mixtures low in leucine are given in figure 1. In the right half of table 3 these values are summarized and the probabilities that the differences between adjacent entries

TABLE 3

Nitrogen balance index of ingested nitrogen (K')

USING N-FREE AND FULL-N DIETS				USING HALF-N AND FULL-N DIETS			
Expt. no.	Amino acid mixture	K'	P [1]	Expt. no.	Amino acid mixture	K'	P [1]
I	Complete	1.29		V	Complete	0.95	
			> 0.6				> 0.9
V	Complete	1.27		I	Complete	0.94	
			> 0.5				> 0.9
IV	⅓ DL-leucine	1.24		II	⅓ L-leucine	0.95	
			> 0.06				> 0.01
II	⅓ L-leucine	1.12		IV	⅓ DL-leucine	0.79	
			> 0.09				> 0.01
III	⅙ L-leucine	1.03		III	⅙ L-leucine	0.64	

[1] P = probability that the indicated difference arose from chance alone as computed by the Student "t" test.

are due to chance are indicated. This method of comparison makes the one-third L-leucine amino acid mixture equal to the "complete" mixture and superior to the one-third DL-leucine mixture. The latter is in turn superior to the one-sixth L-leucine mixture.

The one-third DL-leucine mixture contains the same amount of L-leucine as the one-sixth L-leucine mixture plus an equal amount of D-leucine. The K' values indicate that D-leucine can be utilized to meet part of the requirement of L-leucine. The one-third DL-leucine mixture contains the same amount of total leucine as the one-third L-leucine mixture but in the racemic mixture half is present in the D-form. The poorer

utilization of the one-third DL-leucine mixture indicates that D-leucine is less efficiently utilized than L-leucine.

Rose ('38, '49) has reported that D-leucine is not utilized in the absence of L-leucine in rat or man. Grau and Peterson ('46) have found that DL-leucine is utilized by the growing chick just as efficiently as the pure natural isomer. Ratner, Schoenheimer and Rittenberg ('40) fed D-leucine, labelled with isotopic N and H, together with casein to the rat and found that the carbon chain of administered D-leucine is converted fairly directly to L-leucine but that the N is exchanged. The results of the present investigation and the above-mentioned findings can be harmonized by postulating that D-leucine can be utilized to form L-leucine in the presence, but not in the absence, of a small supply of dietary L-leucine.

It would be highly desirable to determine whether the old method, using the N-free and full-N diets, or the new method, using the half-N and full-N diets, gives the better test of quality of amino acid mixtures. In reproducibility of mean results for the same diet the two methods are substantially equal, both giving good agreement between experiments I and V. The fact that the three low-leucine amino acid mixtures are each significantly different from the other by the new method, whereas only one comparison among the three gives a significant difference by the old method, and the more reasonable magnitude of K' values obtained by the new method, tend to favor the latter. Only further experiments will prove whether nitrogen utilization values determined from half and full maintenance nitrogen requirement diets are better than similar values based on a N-free diet. The present experiments show that it is feasible to determine nitrogen balance indexes by both methods, and illustrate some objections to the use of nitrogen excretion values from N-free dietary periods.

Nitrogen requirement for nitrogen equilibrium

By extrapolation or interpolation of the half-N to full-N line of each experiment in figure 1 to its intersection with

the zero nitrogen balance line, an estimate of the nitrogen intake required for nitrogen equilibrium (NI_e) was obtained. This maintenance requirement of nitrogen is an inverse measure of the quality of an amino acid mixture. The values obtained are listed at the bottom of table 2. The NI_e for the complete amino acid mixture was much smaller in experiment V than in experiment I. By contrast, the K' values from these experiments were equal. It may be that the rats experienced a progressive protein depletion which increased as the experiment progressed, in spite of body weight maintenance. Protein depletion causes a reduction in the requirement of nitrogen for maintenance of equilibrium which is usually accompanied by an increase in utilization index (Allison, Seeley, Brown and Anderson, '46). In previous experiments in this laboratory the greatest decrease in the maintenance requirement of nitrogen observed in a group of rats was 7%, which was not a statistically significant decrease. In the data here presented the maintenance requirement of nitrogen is not so consistent an index of the quality of an amino acid mixture as K'.

Energy metabolism

On the 6th and 7th day of each experimental diet period, and at the end of each experiment after a 36-hour fast, the resting energy metabolism was determined. Each heat production value was the average for all the rats for three quiet periods, each an hour long, distributed uniformly in a 10-hour experiment. On those days when the experimental diet was given one of the quiet periods fell in each of the thirds of the 12-hour interval between feedings. The heat production values may therefore be considered to represent the resting heat production of the daylight half of a day. The methods used were similar to those described earlier (Anderson and Nasset, '48). The principal changes from the earlier methods consisted in using a half-gallon fruit jar to house each rat, with nothing to restrict movement, and in providing extra illumination during the quiet periods.

The average basal heat production was 18.6 Cal./day/rat and the average resting heat production after feeding the complete amino acid mixture full-N diet was 25.8 Cal./day/rat. The heat production values obtained after feeding the other diets were not significantly different in any case from those obtained after feeding the complete amino acid mixture. It is concluded that any alteration in heat production which may have resulted from the reduction of leucine in the amino acid mixture was so small that it was obscured by other causes of variation in the measurement of heat production. The coefficient of variation of a mean heat production value for 6 rats was 2.5%, which corresponds to a coefficient of variation of 6% for the heat production of a single rat.

SUMMARY AND CONCLUSIONS

Nitrogen balance and energy metabolism studies were performed on adult rats receiving diets containing mixtures of pure amino acids as the source of nitrogen. Each experiment included a 7-day period on a N-free diet, a 7-day period on an amino acid mixture at approximately half the maintenance level of nitrogen, and a 7-day period on the same amino acid mixture in about full maintenance quantity. These diets were fed by stomach tube twice daily so that each rat received the same quantity of diet every day.

Stepwise reduction in the amount of leucine to one-sixth that in egg protein failed to alter the heat production significantly.

The nitrogen balance index of dietary nitrogen (K' of Allison and Anderson, '45) was computed for each amino acid mixture in two ways; first, using the N-free period as a reference and second, using the half-N period as a reference.

The half-N reference method of determining K' seems preferable to the N-free reference method for the following reasons: (1) the former method gives K' values of reasonable magnitude, i.e., slightly less than unity, as compared with the latter method which gives some K' values well above unity; (2) rats will not voluntarily eat a N-free diet in full

quantity for 7 days. Further experiments are needed to prove which method gives the more consistent measure of the quality of an amino acid mixture or protein.

The half-N reference method of computation leads to the following two conclusions which are not confirmed by the N-free reference method: (1) the leucine content of the one-third L-leucine amino acid mixture, which is 21.1 mg L-leucine nitrogen per gram of total nitrogen (one-third the proportion in whole egg protein), is adequate to permit complete utilization of the whole mixture; (2) as a supplement to the one-sixth L-leucine amino acid mixture L-leucine is more efficiently utilized than D-leucine.

Both criteria support the following conclusions: (1) the quantity of leucine in the one-sixth L-leucine mixture (10.6 mg L-leucine nitrogen per gram of total nitrogen, which is one-sixth the proportion in whole egg protein) is suboptimum; (2) the one-third DL-leucine amino acid mixture, which contains in addition to the above-mentioned quantity of L-leucine an equal quantity of D-leucine, is significantly better than the one-sixth L-leucine mixture. D-leucine is utilizable, therefore, to replace part of the L-leucine required for N equilibrium in the adult rat.

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